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# EFFECT OF ANDROGEN AND ESTROGEN ON SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE OF RAT PROSTATE AND SEMINAL VESICLE[1]

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PREVIOUS work in this laboratory indicates that in ovarian tissues correlations can be made between the functional state of the tissues and fluctuations in activity of such enzymes as succinic dehydrogenase (SDH-ase) (Meyer, Soukup, McShan, Biddulph, 1947), malic dehydrogenase and cytochrome oxidase (McShan, Erway, Meyer, 1948), adenosine triphosphatase (Biddulph, Meyer, McShan, 1946), and acid and alkaline phosphatase (Stafford, McShan, Meyer, 1947). The experiments to be reported here were undertaken to determine whether such a relationship exists in the prostate and seminal vesicle with respect to SDH-ase and cytochrome oxidase following castration and replacement therapy.

## MATERIALS AND METHODS

The animals used were Sprague-Dawley male rats ranging from 200 to 300 gm. body weight. Of a total of 55 animals, 7 were set aside as normal controls and did not receive any treatment. One animal was used for each experiment; 5 for SDH-ase controls and 2 for cytochrome oxidase. The remainder were castrated and divided into 3 groups.

Group I consisted of 20 animals which did not receive any injections and were sacrificed at intervals of 1, 4, and 6 days after castration. In the 1-day category, sufficient tissue was obtained from one animal for one experiment, but on the 4th and 6th days after castration atrophy of the prostate was great enough to require combining the glands from 2 animals.

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Group II contained 14 animals which received 2 subcutaneous injections per day, 12 hours apart. Each injection consisted of 300 $\mu$g. testosterone propionate dissolved in 0.2 cc. of corn oil. The first injection was given at the time of castration and the animals were sacrificed at intervals of 1, 4, and 6 days after castration.

Group III consisted of 14 animals which received the same dosage of testosterone propionate as group II plus simultaneous injections of 100 $\mu$g. of estradiol dipropionate dissolved in 0.2 cc. of corn oil. As in group II, the first injections were given at the time of castration and the animals were sacrificed at 1, 4, and 6 days after castration.

After killing the animals by cervical dislocation the ventral lobe of the prostate and both seminal vesicles were dissected out. The tissues were thoroughly chopped with a sharp scalpel blade, rinsed in ice-cold glass-distilled water, blotted dry on analytical grade filter paper and weighed. This procedure was followed in order to remove as much of the glandular secretions as possible. Pieces of tissue weighing 50 to 200 mg. were homogenized and diluted with glass-distilled water to make either 5% or 1% homogenates, the material being kept ice-cold throughout.

The procedures described by Potter and Schneider (1942) and Schneider and Potter (1943) for SDH-ase and cytochrome oxidase were used in these experiments. The 5% homogenates were used for SDH-ase determinations, but due to the high activity of the tissues 1% homogenates had to be used for cytochrome oxidase. The results of each determination were calculated using the dry weight of the homogenate used for that determination. Results are given in terms of $Q_{O_2}$, or mm$^3$ of oxygen consumed/mg. of dry tissue/hour. Duplicate flasks were used in making all determinations in the Warburg apparatus.

## RESULTS AND DISCUSSION

Examination of Figs. 1 and 2 shows that, following castration, the decline in SDH-ase activity roughly parallels the loss in gland weight. The weight loss in the prostate is fairly uniform between 0 and 6 days while the enzyme activity curve shows no change on day 1 and a sharp drop between days 1 and 4. In the seminal vesicle the weight loss is fairly uniform between days 0 and 6 and the decline in enzyme activity is almost linear between days 0 and 4. Thus the SDH-ase appears to react to an absence of androgen more quickly in the seminal vesicle than in the prostate.

A comparison of these results with the observations of Moore, Price, and Gallagher (1936) and Moore, Hughes, and Gallagher (1936) on the morphological changes in the prostate and seminal vesicle following castration, provides an interesting correlation. These authors found that within 24 hours after castration the height of the secretory cells of the seminal vesicle was reduced to nearly one half of the normal value and almost no secretory granules remained in the cytoplasm. Similar changes occurred in the secretory cells of the prostate but not until the fourth day following castration. Simultaneously with these cytological changes there was a decrease in the size of the lumina of the prostatic tubules and definite changes in the appearance of the

secretion in the seminal vesicle. Changes in the mitochondria occurred but were indefinite. Our data show that there occurs a decline in enzyme activity at about the same time as the loss of secretory granules from the epithelial cells of these tissues and a weight decrease which,

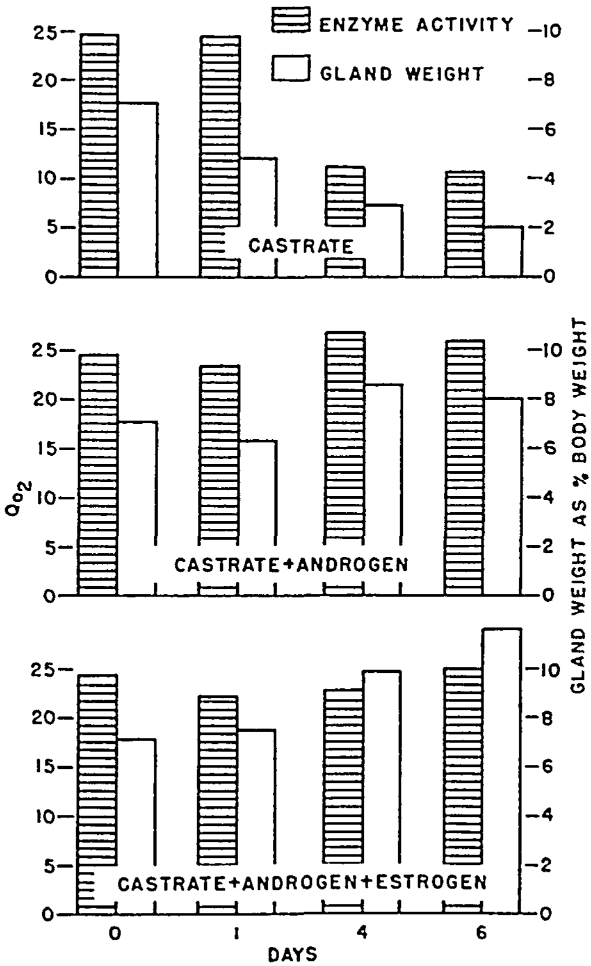


Fig. 1. Effect of testosterone propionate and estradiol dipropionate on the weight and succinic dehydrogenase activity of the prostate.

at least in the case of the seminal vesicle, follows closely the involution of the secretory epithelium.

The ability of the synthetic androgens to maintain the functional and morphological integrity of the male sex accessories in the absence of the testes is well known. Our data show that testosterone propionate, in an approximately physiological dose, will likewise maintain the SDH-ase activity of the accessories at a normal level. The dosage used did not prevent a 23% loss in weight of the prostate during the first day but did prevent such loss in the seminal vesicle.

While the enzyme activity showed a slight increase over normal in both tissues, the increase was somewhat greater and more constant in the seminal vesicle. The observations of Moore *et al.* discussed above and those of Korenchevsky, Dennison and Brovsin (1936) indicate that on a morphological basis the seminal vesicle is more sensitive to the presence or absence of androgen than the prostate. The data presented here show that the same is true with respect to SDH-ase activity.

The simultaneous administration of estrogen and androgen produced striking effects on the gland weight. The prostate showed a

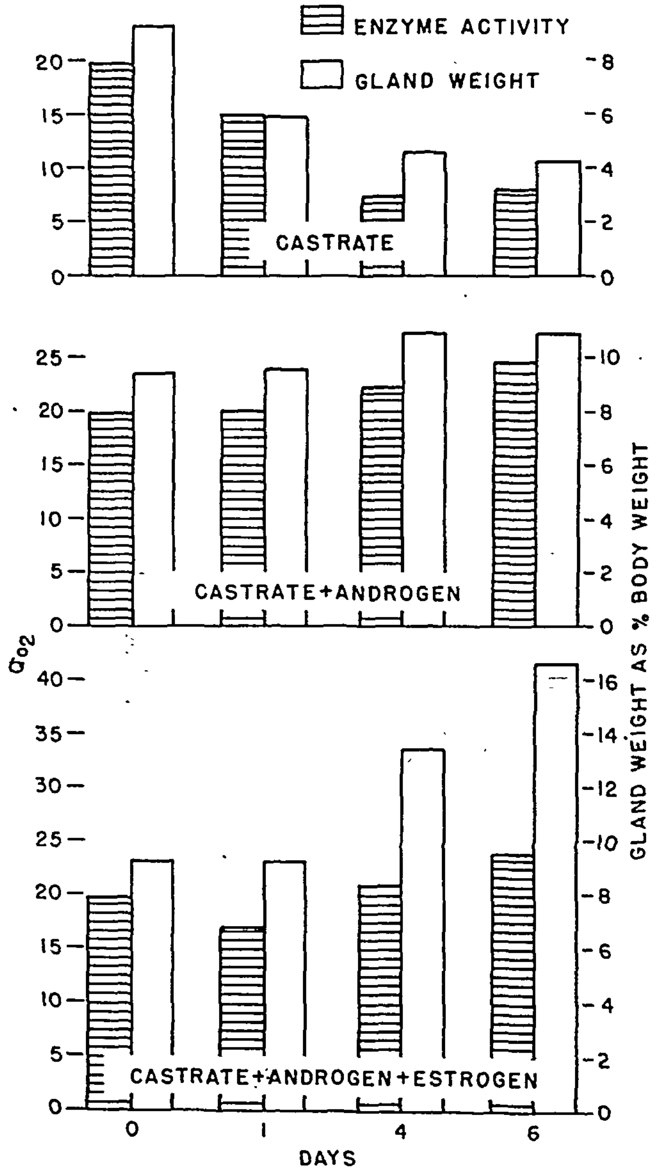


FIG. 2. Effect of testosterone propionate and estradiol dipropionate on the weight and succinic dehydrogenase activity of the seminal vesicle.

slight depression on day 1, not as great, however, as with androgen alone, followed by an almost linear increase to 41% above normal on day 6. The weight of the seminal vesicle underwent no change on day 1 but increased linearly to 77% above normal on day 6.

There is excellent evidence that estrogens stimulate growth of the fibromuscular layer in the male accessories and to a slight extent that of the secretory epithelium, especially in the seminal vesicle (Moore, Hughes and Gallagher, 1936; Korenchevsky, Dennison and Brovsin, 1936; Korenchevsky and Dennison, 1935; Overholser and Nelson, 1935; Tislowitz, 1939). This appears to be a logical explanation for the greater part of the weight increase which we found in this group of animals. However, consideration of the weight changes for tissues treated with androgen alone indicates that the androgen was responsible for some of this increase.

The combined effect of estrogen and androgen on enzyme activity was quite different from that exerted on gland weight. In the prostate, activity was depressed 10% below normal until day 6 when it returned to the normal level. In the seminal vesicle there was a 14% decrease in activity on day 1 followed by a linear rise to 21% above normal on day 6.

Comparison of these results with those obtained using androgen alone reveals that, in the seminal vesicle, the activity is no greater on day 6 than that obtained with androgen alone. On day 1, however, there was a definite depression which was not obtained with androgen alone. In the prostate the activity remained below that obtained with androgen alone throughout the experimental period. However, reference to Table 1 will show that the ranges of values obtained for these two groups overlap in several places. Hence the significance of these differences is open to question and one hesitates to conclude that androgens and estrogens are antagonistic in their effect on male accessories. The data on gland weights in these two groups are open to the same criticism except for the 6-day seminal vesicles; here the results indicate a definite synergism between the two hormones.

The cytochrome oxidase data, though not extensive, suggest that the activity of this enzyme follows much the same general pattern as SDH-ase. This might be expected since cytochrome oxidase is a part of the SDH-ase system and previous work in this laboratory has shown this to be true in other endocrine tissues.

## SUMMARY

The effects on the seminal vesicle and prostate of castration, castration followed by androgen injections, and castration followed by androgen and estrogen injections have been studied in the rat. Changes in activity of succinic dehydrogenase, cytochrome oxidase, and gland weights were determined for each of these conditions and compared

TABLE I. SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE ACTIVITY OF RAT PROSTATE AND SEMINAL VESICLE

| | | Succinic dehydrogenase | | | | | | Cytochrome oxidase | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 Day | | 4 Days | | 6 Days | | 4 Days | | 6 Days | |
| Treatment | | Prostate | Sem. Ves. | Prostate | Sem. Ves. | Prostate | Sem. Ves. | Prostate | Sem. Ves. | Prostate | Sem. Ves. |
| None | $Q_{O_2}$ | 24.4 (23.1–27.5) | 19.8 (16.5–22.8) | | | | | 274 (257–291) | 147 (142–152) | | |
| | Weight[2] % | 7.16 (6.32–8.44) | 9.3 (6.7–12.0) | | | | | 6.9 (6.3–7.4) | 9.4 (9.2–9.6) | | |
| | Weight mg. | 200 (158–249) | 235 (197–285) | | | | | 172 (158–186) | 235 (229–241) | | |
| | No. Expts. | 5 | 5 | | | | | 2 | 2 | | |
| Castrate | $Q_{O_2}$ | 24.1 (18.2–29.3) | 15.0 (11.9–18.2) | 11.1 (9.4–13.3) | 7.3 (4.6–10.2) | 10.9 (8.5–14.1) | 7.9 (5.6–11.9) | 190 | 95 | 190 | 75 |
| | Weight % | 4.8 (3.8–6.4) | 5.9 (4.4–7.6) | 2.9 (2.4–3.1) | 4.6 (3.6–5.5) | 2.0 (1.2–3.1) | 4.3 (3.6–5.7) | 3.1 | 5.2 | 1.9 | 3.8 |
| | Weight mg. | 133 (89–191) | 163 (104–226) | 94 (58–78) | 102 (55–155) | 58 (34–89) | 106 (50–167) | 88 | 147 | 50 | 103 |
| | No. Expts. | 4 | 4 | 4 | 4 | 3 | 3 | 1 | 1 | 1 | 1 |
| Castrate plus androgen | $Q_{O_2}$ | 23.5 (20.5–25.6) | 20.0 (17.6–22.0) | 26.9 (25.2–27.5) | 22.1 (17.2–26.0) | 26.0 (21.4–29.8) | 24.4 (21.8–27.4) | 284 | 109 | 291 | 112 |
| | Weight % | 6.3 (3.6–8.3) | 9.5 (7.4–10.6) | 8.6 (5.8–13.3) | 10.8 (7.3–15.7) | 8.0 (5.2–12.5) | 10.8 (8.8–11.9) | 5.8 | 7.2 | 5.2 | 11.1 |
| | Weight mg. | 150 (96–190) | 224 (200–243) | 298 (210–360) | 377 (277–432) | 206 (150–237) | 301 (270–338) | 200 | 253 | 182 | 390 |
| | No. Expts. | 4 | 4 | 4 | 4 | 5 | 5 | 1 | 1 | 1 | 1 |
| Castrate plus androgen plus estrogen | $Q_{O_2}$ | 22.0 (20.0–25.5) | 17.0 (15.9–19.2) | 22.6 (19.6–24.5) | 20.9 (18.1–22.0) | 24.9 (21.9–28.8) | 23.9 (20.5–27.5) | 288 | 162 | 331 | 210 |
| | Weight % | 7.4 (6.9–8.0) | 9.3 (6.8–12.3) | 9.8 (7.4–13.8) | 13.3 (12.2–15.2) | 11.6 (9.5–12.7) | 16.6 (13.9–18.7) | 13.7 | 12.1 | 12.7 | 13.9 |
| | Weight mg. | 189 (183–200) | 234 (179–295) | 220 (177–281) | 328 (304–365) | 250 (228–292) | 391 (367–412) | 440 | 389 | 394 | 432 |
| | No. Expts. | 4 | 4 | 5 | 5 | 4 | 4 | 1 | 1 | 1 | 1 |

[2] Values in this line were calculated as $\frac{\text{gland weight}}{\text{body weight}} \times 10^{-4}$.

to the normal. Castration caused a significant decrease in both enzyme activity and gland weight while the administration of androgen maintained these factors at the normal level. The simultaneous administration of androgen and estrogen caused a significant rise above normal of both factors in the seminal vesicle but only of the gland weight in the prostate. The possible relationship of these findings to the morphology and function of the glands is discussed.

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# OBSERVATIONS ON THE EFFECTS OF PREPARTAL AND POSTPARTAL ESTROGEN AND PROGESTERONE TREATMENT ON LACTATION IN THE RAT

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NUMEROUS attempts to determine the relation of hormones to the initiation of lactation following parturition have been made. The onset of lactation has been attributed to removal of an inhibitory action of progesterone, estrogen or a combination of progesterone and estrogen. The establishment of lactation following the removal of corpora lutea in the rabbit (Hammond, 1917) and following the removal of luteinized ovaries in the rat (Selye, Collip and Thomson, 1933) suggested an inhibitory action of the secretion of the corpora lutea but injection of extract of the corpora lutea (Anselmino and Hoffmann, 1936) and progesterone (Folley and Kon, 1937a) in lactating rats failed to inhibit established lactation. The report of Parkes and Bellerby (1927) demonstrated an inhibitory action of estrogens on established lactation in the rat with intact ovaries. Folley and Kon (1937b), Edelmann and Gaunt (1941) and Barsantini and Masson (1947) have shown that inhibition of established lactation by estrogens is more effective in normal than in ovariectomized rats. Fauvet (1941) found that simultaneous injection of estrogen and progesterone produced marked inhibition of established lactation in ovariectomized rats while estrogen alone induced no inhibition.

Two studies have been made on the effect of prepartal injection of estrone on lactation. Smith and Smith (1933) found that the initiation of lactation was prevented in the rabbit by estrone administration begun on the 20th day of pregnancy. Edelmann and Gaunt (1941) reported that estrone treatment started 4 days before delivery in the normal rat did not prevent initiation of lactation although it brought about the inhibition of established lactation a few days earlier than did similar treatment started on the day of delivery. We are not aware of any observations on the effect of prepartal administration of progesterone or of a combination of progesterone and estrogen on the initiation of lactation. Therefore, the present study was planned to observe the effects of prepartal injection of estrogen alone, progesterone alone

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and various combination of these hormones on the initiation of lactation in the rat and to compare these results with the effects obtained by similar hormone treatment after the establishment of lactation.

## METHODS

Daily subcutaneous injections were given to the mothers and the growth rate of the suckling young was used as the index of the rate of lactation (cf. Cowie and Folley, 1947). Uniparous rats were used in all experiments and six rats per litter were provided at the beginning of the lactation period, where the lactation period is defined as beginning at delivery. In the ovariectomized cases the operation was carried out on the day of parturition or the first day thereafter under ether anaesthesia. In those animals showing prolonged pregnancy as a result of prepartal hormone treatment the young were removed on the 22nd day of pregnancy through a mid-line incision by hysterectomy under ether or local procaine anaesthesia. Days of pregnancy were counted from the day that sperms were found in the vaginal smear. The duration of pregnancy was normally 22 days in the strain of rats used in this study.

## RESULTS

The growth rates of 85 litters containing 6 young rats per litter were observed in this study. The mothers of 63 of these litters were given hormone injections; the mothers of 22 litters were untreated controls. The average weights of the young are shown in Table 1. The living litters included in the table are those with all 6 members surviving, it being expected that the death of even 1 member would alter the supply of milk for the remaining members.

*Effects of estrogen.* Daily injection of 1 mg. of diethylstilbestrol dipropionate begun on the second day of lactation brought about a marked decrease of lactation in 4 normal and in 4 ovariectomized rats while treatment with 200$\gamma$ of this substance per day inhibited lactation in 4 normal animals although it did not produce inhibition in 3 out of 4 ovariectomized rats (Table 1, section 1). All mothers treated with diethylstilbestrol dipropionate and those given 200$\gamma$ of estrone lost weight during the lactation period. All other treated mothers and the untreated controls gained weight.

The results in section 2 of Table 1 show that established lactation was not inhibited in 3 ovariectomized rats given 200$\gamma$ or in 4 ovariectomized rats given 100$\gamma$ of estrone daily from the second to the 16th day of lactation, while a variable extent of inhibition was obtained in normal animals during daily injection of either 100$\gamma$, 25$\gamma$ or 5$\gamma$ of estrone. The onset of inhibition occurred 10 to 12 days after the beginning of estrone administration.

Estrone treatment begun before parturition did not prevent the initiation of lactation though depression of established lactation did occur about the 10th to 12th day after parturition in all mothers showing well formed corpora lutea (Table 1, section 3). In one animal injection of 100$\gamma$ of estrone daily was begun on the 20th day of preg-

nancy. Although the young were delivered 1 day premature they were all viable and lactation was promptly established. The young were growing at a normal rate up to the 6th day of lactation when they were killed by the mother. In an effort to avoid premature delivery the daily dosage of estrone was reduced to 25γ and injection was begun on the 19th day of pregnancy in 3 rats. The first of these failed to give birth to the young and died in labor. Twelve hours after the second animal began to show bleeding and labor movements the young were removed by hysterectomy. Injection of estrone was continued and on the 6th day after the operation normal lactation was established and continued normally up through the 14th day but thereafter lactation was markedly depressed. In the third animal the young were delivered by hysterectomy 24 hours after the first 25γ injection of estrone; they were premature and did not survive. This animal accepted the young from a foster mother and lactation was promptly established. It should be pointed out that the ovaries were removed from this last animal at the time of hysterectomy and that lactation continued normally throughout the period of estrone treatment. Daily injection of 25γ of estrone was started on the 21st day of pregnancy in 4 rats. Although initiation of lactation was not prevented, marked depression of lactation occurred in 3 of these 4 rats about the 10th day after delivery. The other animal showed no depression of lactation throughout the experiment. It is interesting to note that large ovaries with well-developed corpora lutea (fig. 5) were found in the 3 animals showing depression of lactation and that small ovaries with atrophic corpora lutea (fig. 6) were found in the rat showing no depression of lactation.

*Effects of progesterone.* Neither 2 mg. nor 5 mg. doses of progesterone per day inhibited lactation in intact rats when treatment was started on the second day of lactation (Table 1, section 4). To arrive at a dosage of progesterone adequate for study of the effects on initiation of lactation the amount of this hormone required to prevent the onset of parturition was determined. It was found that parturition never occurred, although some uterine bleeding was seen, in 3 rats given 1 mg. of progesterone daily beginning on the 19th day of pregnancy. Labor was observed in 1 of these animals. When the dosage was increased to 2.5 mg. no uterine bleeding appeared and the animals showed no signs of labor. The young were delivered by hysterectomy on the 22nd day of pregnancy and daily injections were continued for 10 days after the operation. The lactation rate was approximately the same as that of control animals similarly operated but not treated with progesterone (cf. sections 5 and 10 of Table 1). The slow rate of growth observed in the young of both groups of operated animals during the first 5 postoperative days is apparently due to operative trauma.

*Effects of simultaneous treatment with estrone and progesterone.* In 3

TABLE 1. MEAN VALUES ON THE GROWTH RATES OF YOUNG IN LITTERS CONTAINING 6 MEMBERS DURING VARIOUS TREATMENTS OF THE MOTHER

| Daily treatment of mother | | | | Condition of mother | No. of litters | Initial wt. | 5th day | | 10th day | | 13th day | | 16th day | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Substance | and section | Dose | Period | | | | wt. | litters living† | wt. | litters living† | wt. | litters living† | wt. | litters living† |
| | | | | | | gm. | gm. | % | gm. | % | gm. | % | gm. | % |
| S | 1 | 1 mg. | 2L–16L | N | 4 | 6.1 | 9.3 | 100 | 11.7 | 75 | 13.6 | 50 | 15.9 | 25 |
| | | 1 mg. | 2L–16L | O | 4 | 5.7 | 9.0 | 100 | 12.3 | 100 | 14.5 | 75 | 17.4 | 50 |
| | | 200γ | 2L–16L | N | 4 | 6.3 | 9.3 | 100 | 12.4 | 75 | 11.6 | 75 | 15.7 | 25 |
| | | 200γ | 2L–16L | O | 4 | 6.3 | 10.3 | 100 | 16.1 | 75 | 19.1 | 75 | 21.3 | 75 |
| E | 2 | 200γ | 2L–16L | O | 3 | 6.0 | 9.8 | 100 | 14.1 | 100 | 17.5 | 100 | 21.2 | 100 |
| | | 100γ | 2L–16L | N | 2 | 6.6 | 10.3 | 100 | 13.2 | 100 | 17.6 | 50 | 18.8 | 50 |
| | | 100γ | 2L–16L | O | 4 | 6.9 | 9.9 | 100 | 16.2 | 100 | 20.0 | 100 | 22.8 | 100 |
| | | 25γ | 2L–16L | N | 3 | 6.7 | 12.2 | 100 | 18.5 | 100 | 18.7 | 67 | 18.0 | 67 |
| | | 5γ | 2L–16L | N | 2 | 6.0 | 9.3 | 100 | 12.3 | 100 | 13.0 | 100 | 13.1 | 100 |
| E | 3 | 100γ | 20P– 5L | S.D. | 1 | 5.1 | 8.9 | 100 | | | | | | |
| | | 25γ | 19P–16L | H | 1 | 6.6 | 7.4 | 100 | 13.9 | 100 | 18.3 | 100 | 18.0 | 100 |
| | | 25γ | 19P–16L | H and O | 1 | 7.3 | 12.0 | 100 | 18.7 | 100 | 22.7 | 100 | 26.6 | 100 |
| | | 25γ | 21P–14L | S.D. | 3 | 6.4 | 13.2 | 100 | 18.2 | 100 | 18.7 | 67 | | |
| | | 25γ | 21P–16L | S.D.* | 1 | 6.6 | 11.5 | 100 | 16.9 | 100 | 19.5 | 100 | 24.2 | 100 |
| P | 4 | 2 mg. | 2L–16L | N | 3 | 6.5 | 10.9 | 100 | 17.1 | 100 | 20.5 | 100 | 24.6 | 100 |
| | | 5 mg. | 2L–16L | N | 2 | 6.3 | 10.2 | 100 | 16.7 | 100 | 19.4 | 100 | 21.8 | 100 |
| P | 5 | 2.5 mg. | 19P–10L | H | 3 | 6.1 | 6.9 | 100 | 12.0 | 100 | 15.6 | 100 | 19.3 | 100 |
| E P | 6 | {100γ 2.5 mg. | 2L–16L | O | 3 | 6.4 | 10.9 | 100 | 16.0 | 100 | 18.2 | 100 | 18.3 | 100 |
| E P | | {25γ 2.5 mg. | 2L–16L | O | 4 | 6.5 | 11.5 | 100 | 17.8 | 100 | 20.2 | 100 | 20.4 | 100 |
| E P | | {10γ 2.5 mg. | 2L–16L | O | 7 | 6.5 | 9.8 | 100 | 15.3 | 100 | 17.5 | 100 | 17.9 | 100 |
| E P | 7 | {100γ 1 mg. | 20P–16L | H and O | 2 | 6.7 | 9.1 | 100 | 14.7 | 100 | 17.6 | 100 | 20.0 | 100 |
| E P | | {25γ 2.5 mg. | 20P–16L | H and O | 2 | 6.1 | 7.7 | 100 | 11.4 | 100 | 13.2 | 100 | 14.3 | 100 |
| none | 8 | | | N | 13 | 6.4 | 10.6 | 100 | 17.4 | 100 | 21.1 | 100 | 25.2 | 100 |
| none | 9 | | | O | 7 | 6.1 | 9.5 | 100 | 15.8 | 100 | 19.6 | 100 | 23.8 | 100 |
| none | 10 | | | H | 2 | 6.0 | 7.1 | 100 | 12.2 | 100 | 16.2 | 100 | 20.1 | 100 |

S = diethylstilbestrol dipropionate.
E = estrone.
P = progesterone.
2L = second day of lactation.
20P = twentieth day of pregnancy.

N = intact ovaries.
O = ovariectomized.
S.D. = spontaneous delivery.
H = removal of young by hysterectomy.

* This animal showed atrophic ovaries at autopsy (see fig. 6).
† Litters with all members surviving.

ovariectomized rats daily amounts of progesterone (2.5 mg.) and estrone (100γ), without effect when given separately, were injected simultaneously, beginning on the 2nd day of lactation. The data in section 6 of Table 1 show that lactation was markedly depressed in these 3 animals after the 13th day. Similar results were obtained when the same dosage of progesterone (2.5 mg. daily) was given in combination with 25γ or with 10γ of estrone.

Two combinations of estrone and progesterone were used for simultaneous injections beginning on the 20th day of pregnancy (Table 1, section 7). Viable young were delivered by hysterectomy 48 hours after the first injection and ovariectomy was done at the same time. In 2 rats injected with 100γ of estrone and 1 mg. of progesterone lactation was established promptly and it was equal to that of the operated controls throughout the period of observation. In 1 animal given 25γ of estrone and 2.5 mg. of progesterone the initiation of lactation was retarded but a slightly subnormal rate was established by the 7th day after the operation, with further inhibition occurring on the 12th day. In another animal receiving 25γ of estrone and 2.5 mg. of progesterone an incrustation of the nipples developed which obstructed their openings. This horny covering was sloughed off on the 6th day after hysterectomy, but lactation was not established until the 13th day although the animal had nursed young from foster mothers almost continuously from the time of the operation. Lactation continued at a normal rate until the 26th day after hysterectomy (13th day of lactation) when marked inhibition occurred. The 13th day after hysterectomy was taken arbitrarily as the first day of lactation for this animal in order to show in the table the effects of continued hormone treatment on established lactation.

*Histological changes in the mammary gland.* Sections of the mammary gland in animals of the various treated groups were made on the 17th to 19th day of lactation. Findings typical of the various groups are shown in figures 1 to 4. The alveolar sacs were large and well filled with milk in the ovariectomized rats given 100γ of estrone and the alveolar cells appeared normal (fig. 1). On the other hand, the alveolar sacs were small and frequently almost devoid of milk in the intact rats given 100γ of estrone and the alveolar cells usually showed proliferation (fig. 2). In the ovariectomized rats given estrone and progesterone simultaneously the alveoli were usually small and the alveolar cells frequently formed walls several layers thick indicating pronounced proliferation (figs. 3 and 4). It should be noted that 25γ of estrone (fig. 4) was as effective as 100γ (fig. 3) in bringing about proliferation when these amounts were given with 2.5 mg. of progesterone.

## DISCUSSION

The results indicate that neither estrogens alone nor progesterone alone inhibits established lactation or prevents the initiation of lacta-

tion. A large number of reports indicates that estrogens inhibit lactation. It is suggested that the findings in many of these studies may be reconciled with our observations that estrone neither prevents nor inhibits lactation in the ovariectomized rat if the following assumptions are made: (1) Inhibition of lactation in estrogen-treated intact animals is due to a synergistic action of the injected estrogen with an ovarian secretion, probably progesterone. (2) Inhibition of lactation in ovariectomized animals requires dosages of estrogen which greatly exceed the normally occurring secretion. (3) the stimulation resulting from nursing or manipulation of the mammary gland and from complete removal of the milk is essential to maximal lactation.

The assumption that inhibition of lactation by estrogen treatment in intact animals is due to a synergism with another ovarian secretion is supported by the observation in this report that simultaneous injection of progesterone and estrone inhibits lactation in ovariectomized rats. Since 5$\gamma$ of estrone per day markedly depresses lactation in intact rats while a 200$\gamma$ dosage shows no inhibitory effect in ovariectomized animals it is clear that the inhibition observed in intact animals is not due to estrogens alone, and it is demonstrated that a synergistic action with some other ovarian hormone is occurring. The failure of 25$\gamma$ of estrone to produce any depression of lactation in a rat showing almost complete absence of corpora lutea (fig. 6) at the end of the lactation period emphasizes the importance of functional corpora lutea to the inhibitory action of estrogen. The histological appearance of the mammary gland of the treated animals indicates that inhibition of lactation in the present experiments is correlated with proliferation of the alveolar tissue. The numerous small alveoli found in the mammary glands 10 to 12 days after the first injection of intact animals treated with estrogen and of ovariectomized rats treated with estrone and progesterone are lined with cells which suggest growth (cuboidal cells) and an abeyance of secretion (diminution or absence of fat globules). Fauvet (1941) observed similar histological changes in the mammary gland of ovariectomized rats treated simultaneously with estrogen and progesterone. It has been suggested that substances which induce mammary growth also bring about inhibition of lactation (Loeb, 1932; Nelson, 1934; Folley and Kon, 1937b; Nelson, Gaunt and Schweizer, 1943). Nelson (1937) has shown that estrone induces complete mammary development in the ovariectomized guinea pig. On the other hand, Nelson (1935) found that although estrone produces lobule proliferation in the ovariectomized rat it does not bring about complete development of the mammary glands. Our data from ovariectomized lactating rats indicate that estrone alone neither inhibits lactation nor stimulates growth of the alveoli, while a combination of estrone and progesterone does both. One of the combinations of estrone and progesterone (100$\gamma$ and 1 mg.) selected for simultaneous injection before parturition is based on the observation of Scharf and

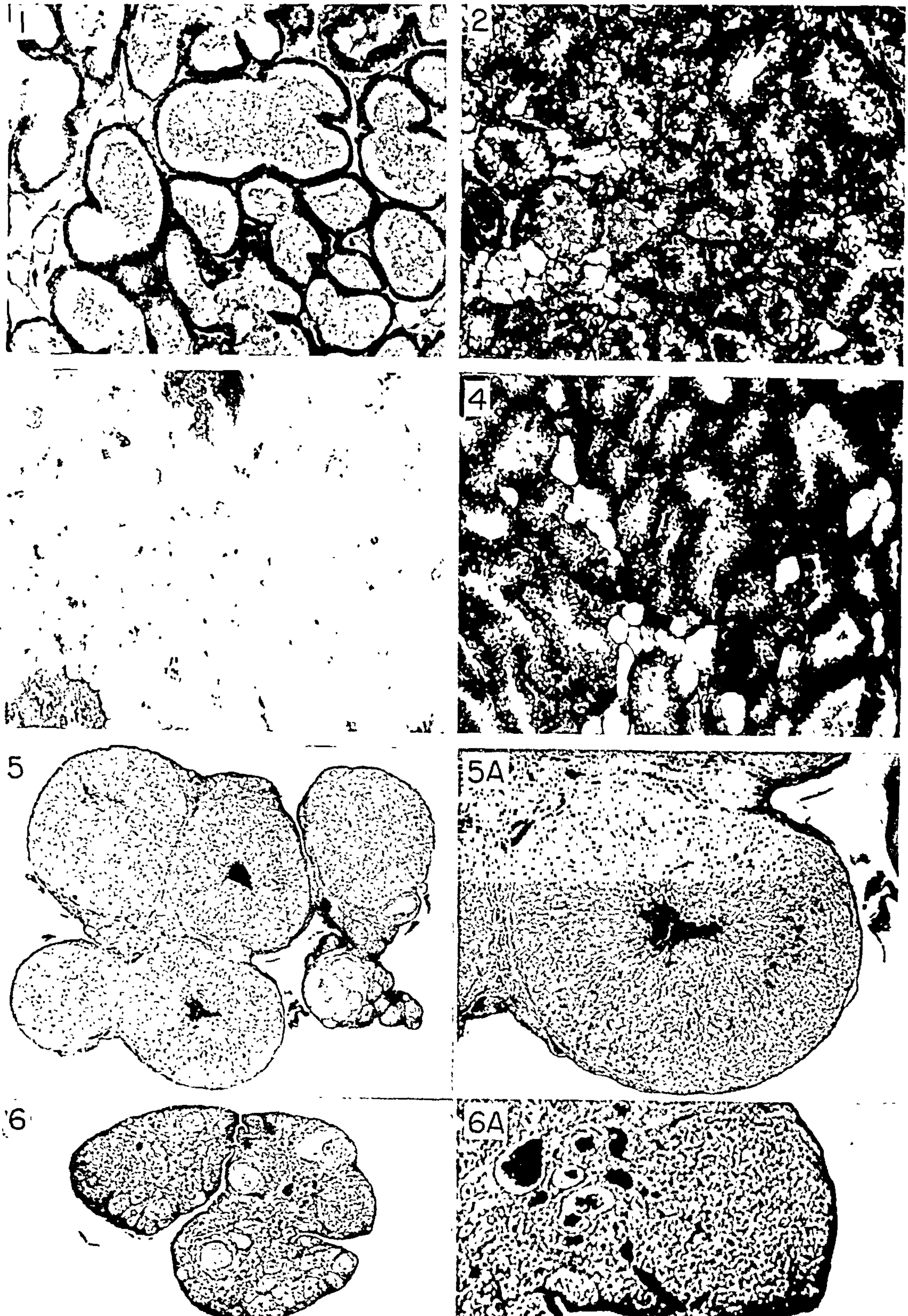


PLATE 1

Lyons (1941) that this combination showed the greatest alveolar development of the 6 combinations tried in immature male rabbits. The other combination (25γ of estrone to 2.5 mg. of progesterone) was used in order to make comparison with results already obtained by postpartal injections. The failure to prevent the initiation of lactation with either of these combinations of estrone and progesterone or with the hormones given separately suggests that removal of an inhibitory influence by reduction of the secretion of ovarian hormones in late pregnancy is not essential to the establishment of lactation provided maximal stimulation of lactogenic processes by nursing and removal of milk is obtained. However, it should be pointed out that it is not known whether the maximal inhibitory effects of these hormones have been attained in the present experiments. In fact, the greater effectiveness of the combination of 25γ of estrone and 2.5 mg. of progesterone in delaying the onset of lactation suggests that a combination of these hormones may be found which would completely prevent the initiation of lactation in the rat even with a maximal stimulus of nursing.

The assumption that inhibition of lactation in ovariectomized animals by estrogen requires dosages which exceed the normally occurring secretion is supported by observations on the rat. In our experiments doses of diethylstilbestrol dipropionate (1 mg. daily) large enough to depress lactation also brought about a marked loss of weight in the lactating animals. The depression of lactation in these rats is perhaps due to toxic effect on the lactating animal rather than to specific inhibition of lactation. Edelmann and Gaunt (1941) found that large doses of estrogen caused lactating rats to lose weight. Trentin and Turner (1941) found that the decrease of food intake in rats is

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EXPLANATION OF FIGURES ON PLATE 1

FIG. 1. Mammary gland removed on the 17th day after parturition from a rat ovariectomized on the day of parturition and injected daily with 100γ of estrone from the 2nd through the 16th day after delivery. ×140.

FIG. 2. Mammary gland removed on the 17th day after parturition from an intact rat injected daily with 100γ of estrone from the 2nd through the 16th day after delivery. ×140.

FIG. 3. Mammary gland removed on the 17th day after parturition from a rat ovariectomized on the day of parturition and injected daily with 100γ of estrone and 2.5 mg. of progesterone from the 2nd through the 16th day after delivery. ×140.

FIG. 4. Mammary gland removed on the 17th day after parturition from a rat ovariectomized on the day of parturition and injected daily with 25γ of estrone and 2.5 mg. of progesterone from the 2nd through the 16th day after delivery. ×140.

FIG. 5. Ovary (showing large corpora lutea) removed on the 17th day after parturition from a rat injected daily with 25γ of estrone from the 21st day of pregnancy through the 16th day after delivery. Compare with 6. ×10.

FIG. 5A. A higher magnification of a corpus luteum shown in figure 5. Compare with 6A. ×22.

FIG. 6. Ovary (showing atrophic corpora lutea) removed on the 17th day after parturition from a rat injected daily with 25γ of estrone from the 21st day of pregnancy through the 16th day after delivery. ×12.

FIG. 6A. A higher magnification of a corpus luteum shown in figure 6. ×33.

proportional to the increase of estradiol benzoate administration.

The extent of the influence of nursing or manipulation of the mammary gland and removal of milk on lactation is not known but it has been demonstrated that the mammary glands of non-nursing rats contain very little milk on the 7th day after parturition (Meites and Turner, 1942a). The prevention of lactation with estrone injection in pseudopregnant and in parturient rabbits by Smith and Smith (1933) was obtained in non-nursing animals and the removal of milk was accomplished manually. Our observation that injection of estrone before parturition in the intact rat does not prevent the initiation of lactation is contrary to the findings of Smith and Smith and in agreement with the observation of Edelmann and Gaunt (1941) in the rat. Nelson (1934) found that estrone given immediately after parturition prevented the initiation of lactation in the intact guinea pig. The failure to prevent the initiation of lactation by estrone administration in the rat suggests a species difference but the possibility that the lack of the stimulus of nursing may have been a factor in the experiments of Smith and Smith should be considered.

The failure of progesterone treatment, begun prepartally, to prevent the initiation of lactation opposes the view advanced by Meites and Turner (1942b) that progesterone might be responsible for the failure of abundant lactation to occur prior to parturition and indicates strongly that the initiation of lactation after parturition does not require the removal of an inhibitory action of progesterone. The failure of postpartal injection of progesterone to inhibit established lactation is in agreement with the findings of Anselmino and Hoffmann (1936) and Folley and Kon (1937a).

## SUMMARY

Observations were made on the effects of prepartal and postpartal administration of estrogens, of progesterone and of combinations of estrone and progesterone on lactation in intact and in ovariectomized rats, using the growth rate of nursing young to indicate the rate of milk secretion.

Daily ($200\gamma$) treatment with estrone or diethylstilbestrol dipropionate begun on the second day of lactation does not inhibit milk secretion in ovariectomized rats while moderate daily ($5\gamma$ and $10\gamma$) injection of estrone inhibits lactation in intact rats, the inhibition appearing 10 to 12 days after the beginning of treatment. The inhibition of lactation induced by treatment with still larger dosage (1 mg.) of diethylstilbestrol dipropionate both in intact and in ovariectomized rats was accompanied by marked loss of weight of the mother and is interpreted as a toxic effect. Prepartal injection of estrone does not prevent the initiation of lactation either in ovariectomized ($25\gamma$) or in intact ($25\gamma$ and $100\gamma$) rats but does produce a delayed depression in intact animals.

Daily treatment with progesterone begun prepartally (2.5 mg.) or postpartally (5 mg.) neither prevents nor inhibits lactation.

Simultaneous injection of estrone and progesterone begun prepartally or postpartally does not prevent lactation but it does inhibit established lactation after 10 to 12 days of treatment.

The marked proliferation of the mammary glands showing inhibition of lactation suggests that stimulation of mammary growth by combined action of estrone and progesterone plays a role in the inhibition of milk secretion in the rat.

ACKNOWLEDGMENTS

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# CHOLINERGIC AND ADRENERGIC COMPONENTS IN THE NEUROHUMORAL CONTROL OF THE RELEASE OF LH IN THE RABBIT

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## INTRODUCTION

The nervous system, in species like the rabbit which do not ovulate spontaneously, controls ovulation by stimulating the anterior lobe of the hypophysis to release lutenizing hormone; LH in turn (alone or in combination with FSH) activates maturation and rupture of the ovarian follicles (Hisaw, 1947). The nature of the mechanism by which the central nervous system exerts its influence on the hypophysis has been the subject of numerous investigations including those of Markee, Sawyer, and Hollinshead (1946–1948). Electrical stimulation experiments by these authors (1946) gave evidence that hypothalamic control of the anterior lobe, which almost certainly involves the pituitary stalk (Brooks, 1940), is not exerted via nerve fibers ending in the pars distalis. The results indicated, rather, that a humoral link, such as the hypophyseal portal system (Wislocki and King, 1936; Green and Harris, 1947), might transport a chemical mediator capable of stimulating the electrically-inexcitable gland cells. Attempts to stimulate LH release by intravascular administration of the neurochemical agents, acetylcholine and adrenaline, led to uniformly negative results (Markee, Sawyer and Hollinshead, 1947, 1948). When adrenaline was introduced directly to the adenohypophysis, however, ovulation was induced in a significant number of cases —5 out of 10 with the optimal concentration (1/1000). Acetylcholine was ineffective even when applied in this direct manner. The presence of an adrenergic component in the natural secretion-stimulus was confirmed (Sawyer, Markee and Hollinshead, 1947) by blocking copulation induced ovulation with Dibenamine, which is a potent adrenolytic agent.

Inasmuch as direct intrahypophyseal administration of acetylcholine failed to stimulate release of LH in the rabbit, as it had also failed in the constant-estrous guinea pig (Dey, 1943), no attempt was made at the time of the Dibenamine study to block the coital stimulus with anti-cholinergic agents such as atropine. Taubenhaus and Soskin (1941) had reported, however, that direct application of acetyl-

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choline to the rat hypophysis results in liberation of luteotrophic hormone. Although the normal hypophyseal control of the corpus luteum in the rabbit appears to be an indirect one (Robson, 1938), it depends upon the integrity of the pituitary stalk (Westman and Jacobsohn, 1940) and might involve a cholinergic mediator. Therefore, to ascertain whether it might interfere with luteal function, we injected atropine intravenously into female rabbits within a minute after copulation. Interestingly enough, ovulation was blocked in a significant number of cases.

The discovery that atropine blocks copulation-induced release of LH implies that the natural neurogenic stimulus includes a cholinergic as well as an adrenergic component. This inference led to a careful study of the time relationships between the atropine-blocked and Dibenamine-blocked components in order to determine whether they function simultaneously or sequentially. In the earlier Dibenamine study the presence of ruptured or hemorrhagic ovarian follicles as observed at laparotomy was employed as the sole criterion of pituitary stimulation. In order to study the effects of partial activation, the present work includes an histological examination of non-ovulating as well as ovulated follicles.

The results of the present study indicate that a cholinergic component of the neurogenic[1] stimulus for the release of LH in the rabbit precedes the adrenergic component and may be responsible for it. Luteal function is not inhibited by either adrenolytic or anti-cholinergic agents, at least not in dosages which block the release of LH. Most of the ovarian follicles whose ovulation has been blocked by atropine or Dibenamine give histological evidence of partial activation. This partial activation includes various degrees of maturation of the ovum, corona formation, secretion of secondary liquor folliculi, and an atypical variety of atresia in which the ovum of a large follicle precedes the granulosa in degeneration. Part of the results of the present study have already appeared in abstract form (Sawyer, Markee and Townsend, 1948).

## MATERIALS AND METHODS

Ninety-four female rabbits were employed in the present investigation. Many were used several times so that the total number of experiments exceeded 160. The average weight was 3.2 kg. and only 2 animals weighed less than 2.4 kg. Most of the rabbits were segregated for weeks before mating though some were used after a few days isolation. Among the latter group, seven were found at laparotomy, 48 hours after mating, to have been pregnant a week or more. None of them ovulated in response to the experimental mating; these cases were excluded from the results of the pharmacological blocking experiments. Also excluded were cases of rabbits whose ovaries were

[1] The term *neurogenic* as used in this paper indicates that the nervous system is involved, but we do not intend it to imply that the stimulus is transmitted by nerve fibers ending in the adenohypophysis.

found to be immature and which failed to ovulate either during the attempted blocking experiment or at subsequent mating two or more weeks later when no drug was employed. It has been shown many times that mating in immature or pregnant females does not result in ovulation (Hammond and Marshall, 1925).

Solutions of atropine and Dibenamine were freshly prepared from the salts[2] on the day in which they were to be used. Dibenamine was dissolved first in absolute alcohol (100 mg./ml.) and then diluted to 10 mg./ml. with unbuffered Ringer-Locke solution. The effective dose, 30 mg./kg., thus required a total volume of 10 ml. for a 3.3 kg. rabbit, and the total solution per animal was injected in about 45 seconds. This dosage was above the minimum lethal dose as observed earlier (Sawyer *et al.*, 1947); in the present work 5 of a total of 41 rabbits which received 30 mg. Dibenamine per kg. expired during the subsequent 16 hours. Atropine was dissolved directly in Ringer-Locke solution and concentrations of 10 mg./ml., 30 mg./ml. and 60 mg./ml. were employed. In preliminary experiments, the results of which will be presented below, dosages of 1, 5, 10, 30, 50, 75 and 100 mg./kg. were injected to determine effective and lethal dosages.

In most of the mating experiments the service of a single vasectomized male was engaged. In a typical experiment the female was placed in a large cage with the male. At the instant after copulation when the male fell off, a stop watch was started and the female hastily removed to an injection box. Injection of the drug into the marginal ear vein was begun immediately or at definite intervals (15 sec., 30 sec., 5 min.) post coitum, and the time at which injection was terminated was also noted. "Rapid" injection experiments eliminated the transferral of the female; one person held her at the termination of coitus while another injected concentrated atropine (60 mg./ml.) as rapidly as possible; only the time of completed injection was recorded in this series.

The ovaries were examined at laparotomy or autopsy usually at 48 hours though occasionally at 24 or 72 hours, after copulation. Unilateral ovariectomy was often performed to obtain histological material for comparison with the remaining ovary, which was to be fixed at autopsy at a later time, under different experimental conditions. Tissues were fixed in Zenker's fluid, sectioned at 10 $\mu$, and stained with Harris' hematoxylin and Everett's (1943) modification of Mallory's tri-acid stain.

The integrity of luteal function was tested by an histological examination of uteri and corpora lutea of animals autopsied at 9 to 14 days after mating, and by the histological confirmation of deciduomata established by threading the uterus at five days post coitum and waiting five or six more days before autopsy (Brouha, 1934).

## RESULTS

*Establishing an optimal dose of atropine.* The two rabbits which received atropine in dosages of 75 and 100 mg./kg. rapidly expired, while they lay on their sides and gasped, and a few apparently

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[2] Atropine sulfate USP XIII (Mallinkrodt) and Dibenamine hydrochloride were used. We are grateful to Dr. William Gump of Givaudan-Delawanna, Inc., for a generous supply of Dibenamine.

spontaneous fasciculations occurred in their otherwise hypotonic muscles. Of 7 animals injected with 50 mg./kg., 2 showed little effect beyond pupillary dilation, 2 required artificial respiration to recover, and 3 died in spite of attempts to resuscitate them. Thus 50 mg./kg. is in the neighborhood of the $LD_{50}$. Preliminary attempts with 30 mg./kg. led to no fatalities but did induce "spontaneous" muscular activity and respiratory embarrassment. This dosage, which was adopted for use in subsequent experiments, has been found to be above the MLD for it was lethal to at least 3 animals of 78 receiving it in the blocking experiments. Lower dosages produced no apparent effect except pupillary dilation, which was induced by as little as 1 mg./kg.

*Blocking experiments with atropine.* The results of atropine-blocking experiments which were conducted independently of Dibenamine experiments are summarized in Table 1. Of 17 rabbits receiving their total dose of 30 mg./kg. within a minute after the termination of

TABLE 1. EFFECT OF ATROPINE SULFATE (30 MG./KG. INTRAVENOUS) ON THE ACTIVATION OF THE RABBIT ADENOHYPOPHYSIS
(Laparotomy at 48 hrs. post coitum)

| Number of animals | Time injection was terminated (relative to copulation) | Ovulated (hypophysis activated) | Failed to ovulate (stimulus blocked from reaching hypophysis) | Percentage blocked by atropine |
|---|---|---|---|---|
| 11 {3 | 2, 4, 9 days before | | 3 | |
| {8 | 1, 2, 2, 5, 8, 8, 9, 12 d. before | 8 | | 27 |
| 17 | 1 minute after* | 8 | 9 | 53 |
| 12 | 5½ minutes after | 11 | 1 | 8 |
| 13 | 10–40 seconds after* | 4 | 9 | 69 |

* These injections were *begun* as soon as possible—certainly less than 15 seconds after copulation (cf. table 2).

coitus, 9 were found at laparotomy 48 hours later to have failed to ovulate. This proportion, 9/17, assumes a high degree of significance in view of the fact that somewhat less than one animal (non-pregnant and apparently mature) in ten fails to ovulate in response to a single mating followed by no drug (unpublished observations). The probability that 9 out of 17 would have failed without the atropine is about one chance in a hundred thousand ($P=0.00001$).

When atropine was withheld for 5 minutes after copulation, however, 11 out of 12 animals ovulated, a figure almost identical to the chance probability without atropine. In these two series a concentration of 30 mg./ml. was employed: the rabbits received 1 ml. solution per kg. weight.

A more rapid injection series, in which a concentration of 60 mg. atropine per ml. was used and the total (30 mg./kg.) dose was in-

jected within 10 to 40 seconds post coitum, contained a higher proportion of failures to ovulate (9/13 or 69%). The four rabbits which did ovulate in this series were observed to be especially "hot." They raised their pelves immediately on being mounted while the others of the series required several seconds or even minutes of the male's efforts before they responded.

No animal which received 30 mg. atropine per kg. would mate later on the same day—the drug presumably threw her out of heat. Of 11 female rabbits that were injected with this dosage of atropine and mated as soon as they returned to heat within twelve days after injection, 3 failed to ovulate. This is not very significantly different from the chance ratio: $P=0.07$.

*Comparative blocking experiments: Atropine vs. Dibenamine.* Neither atropine nor Dibenamine will block ovulation if the injection is delayed many minutes after copulation: almost 100% of the cases ovulated after delayed injections of either drug (see above and Sawyer, Markee and Hollinshead, 1947). The proportion in which pituitary activation is blocked by atropine within one minute post coitum (9/17 or 53%) is low when compared with the results of the one-minute Dibenamine experiments (16/19 or 84%). The fact that an increased percentage of atropine blockage (69%) is achieved by increasing the rapidity of the injection, suggests that the inferiority of atropine to Dibenamine as a blocking agent may rest in temporal factors: the atropine-blocked phenomena may reach completion before the Dibenamine-blocked activities cease.

Before attempting to differentiate more accurately the temporal relationships of the blocking effects, it was desirable to demonstrate the rapidity with which intravenous atropine and Dibenamine reach and evoke a response from effector organs near the hypophysis. The fact that the iris reacts to both drugs, pupillary dilatation or constriction following atropine or Dibenamine, respectively, makes the iris an ideal indicator for such a comparison. Three rabbits were injected with 30 mg. Dibenamine per kg.; the solution was made up as 10 mg./ml., and the injection was delivered at such a speed as to terminate at 45 seconds. The pupils started to constrict, on the average, at 25 seconds, and the constriction was complete at 35 seconds from the beginning of injection. Four rabbits received injections of atropine at identical concentrations, dosages and speeds, with the results that pupillary dilatation began at 21 seconds and was complete at 32 seconds. Thus, it is possible to state that atropine arrives at the base of the brain in sufficient concentrations to induce anti-cholinergic effects at least as rapidly as Dibenamine does to exert its adrenolytic effects. Therefore, Dibenamine cannot be considered the better blocking agent merely by virtue of superior capacity to survive the intravascular journey. It should be noted, in passing, that rapid injections of atropine (dosage, 30 mg./kg.; concentration, 60 mg./ml.; 3

TABLE 2. COMPARATIVE EFFECTS OF ATROPINE AND DIBENAMINE ON THE ACTIVATION OF THE RABBIT ADENOHYPOPHYSIS FOLLOWING COPULATION

| Injection time (seconds after copulation) | | Drug | Number of Animals | Ovulated | Failed to ovulate | Percentage blocked by drug |
|---|---|---|---|---|---|---|
| Begun | Ended | | | | | |
| 30 | 60–90 | Atropine | 10 | 9 | 1 | 10 |
| | | Dibenamine | 10 | 5 | 5 | 50 |
| 15 | 60 | Atropine | 20 | 17 | 3 | 15 |
| | | Dibenamine | 13 | 3 | 10 | 77 |

rabbits) initiated pupillary dilation at less than 15 sec. and dilation was completely by 20 sec. Such rapid injections of Dibenamine are not feasible because they are lethal; furthermore high concentrations of Dibenamine are insoluble in saline even after preliminary solution in alcohol.

To demonstrate more adequately whether the atropine-blocked component of the neurogenic stimulus at copulation actually precedes in time the Dibenamine-blocked component, an attempt was made to find an injection time at which atropine might be completely ineffective and Dibenamine completely effective. The results of these attempts are presented in Tables 2 and 3. They are 4 entirely new series, the experiments of which were initiated after the completion of Table

TABLE 3. DEGREES OF COPULATION-INDUCED PITUITARY ACTIVATION ALLOWED BY DIBENAMINE AND ATROPINE INJECTED AT SIMILAR TIMES IN INDIVIDUAL RABBITS

(Dibenamine, 30 mg./kg., injections started at 15 sec. or later after copulation. Atropine, 30 mg./kg., injections started at 15 sec. or earlier after copulation).

| Rabbit Number | Wgt. Kgms. | Drug | Date | Inject. time, sec. | | Results Ovary, gross and histological appearance |
|---|---|---|---|---|---|---|
| | | | | Begun | Ended | |
| 352 | 3.0 | Diben. | 4/29 | 30 | 60 | Clear foll., no sections |
| | | Atrop. | 5/4 | 15 | 60 | Rupt. +hem. foll.; well luteinized |
| 346 | 3.0 | Diben. | 5/24 | 30 | 90 | Hyperemic foll.; activation atresia = #384 |
| | | Atrop. | 5/4 | 15 | 60 | 9 Rupt. foll.; no sections |
| 318 | 3.1 | Diben. | 5/29 | 15 | 70 | Hyperemic foll.; slight activation = #333 |
| | | Atrop. | 5/4 | 15 | 60 | 9 Rupt. +hem. foll.; no sections |
| 356 | 3.1 | Diben. | 5/6 | 15 | 60 | 4 Rupt foll.; no sections |
| | | Diben. | 6/12 | 15 | 60 | 10 Rupt. foll; well luteinized |
| | | Atrop. | 5/24 | 15 | 60 | 10 Rupt. foll; well luteinized |
| 372 | 2.4 | Diben. | 6/8 | 15 | 60 | Clear foll.; v. slight activation = #372 |
| | | Atrop. | 5/11 | — | 17 | 4 Rupt. +hem. foll.; no sections |
| | | Atrop. | 6/19 | 15 | 60 | 9 Rupt. +hem. foll.; well luteinized |
| 383 | 3.4 | Diben. | 5/24 | 15 | 60 | Hyperemic foll.; activation atresia, Fig. 15 |
| | | Atrop. | 5/29 | 15 | 60 | Hyperemic foll.; slight activation, Fig. 5 |
| 387 | 2.9 | Diben. | 5/24 | 20 | 75 | Clear foll.; v. slight activation = #372 |
| | | Atrop. | 6/2 | 15 | 60 | 11 Rupt. Foll.; well luteinized |
| 384 | 2.9 | Diben. | 6/12 | 15 | 60 | Hyperemic foll.; activation atresia Fig. 12 |
| | | Atrop. | 5/24 | 15 | 60 | 11 Rupt. foll.; well luteinized |
| 333 | 3.3 | Diben. | 6/22 | 30 | 60 | Clear foll.; slight activation, Fig. 4 |
| | | Atrop. | 5/29 | 15 | 55 | 10 Rupt. foll.; well luteinized, Fig. 16 |
| 393 | 3.9 | Diben. | 6/2 | 15 | 60 | Clear foll.; moderate activation, Fig. 6 |
| | | Atrop. | 6/8 | 15 | 60 | 10 Rupt. foll.; well luteinized |

1. It was found that injections beginning at 30 sec. post coitum were 90% ineffective with atropine but only 50% effective with Dibenamine. Actually this latter proportion, 5/10, represents a highly significant degree of blocking effectiveness; with 1/10 as the chance failure the probability that 5/10 will fail without the drug is less than one chance in six hundred ($P = 0.0015$). Nevertheless, a second series, with injections beginning at 15 sec., was undertaken. Under these time relationships atropine was 85% ineffective and Dibenamine was 77% effective. The atropine ratio of 3/20 is still not significantly different from the chance probability ($P > 0.1$), but the Dibenamine ratio of 10/13 would occur by chance less than one time in ten million. All four of the atropine-treated animals which failed to ovulate, as well as most of the Dibenamine-blocked rabbits, gave histological evidence of partial pituitary-activation as will be mentioned below.

An even more significant comparison is presented in Table 3, which employs some of the 15-sec. results of the same animals as those summarized in Table 2. Ten rabbits received, on separate occasions, both atropine (at 15 sec. or earlier) and Dibenamine (at 15 sec. or later). Of these, one rabbit ovulated in spite of the injection of either drug (at exactly 15 sec.); one failed to ovulate following either (at exactly 15 sec.); the other eight ovulated after atropine (at 15 sec. or earlier . . . e.g. #372) and were blocked by Dibenamine (at 15 sec. or later—up to 30 sec.). The ovaries of most of these animals were studied histologically, and considerable evidence of the partial activation of follicles is summarized in Table 3. This phenomenon of partial activation will be considered in detail in the next section.

*Partial activation of follicles in cases of blocked ovulation.* In the rabbit healthy large ovarian follicles do not undergo the maturation process without the mating stimulus. The stages of normal post-coital maturation of the follicle and ovum have been often described (Pincus and Enzmann, 1935, 1937; Waterman, 1943) and will be only briefly outlined here. They include migration of the nucleus of the ovum to the periphery and its flattening there, formation of tetrads, loss of the nuclear membrane, formation of the spindle for and extrusion of, the first polar body, and formation of the spindle for the second polar body. The second polar body is not normally extruded until after ovulation, or rupture of the follicle, which occurs between 10 and 14 hours after mating. While the ovum is maturing, preovulatory changes are occurring in the follicle, including secretion of the secondary follicular liquor (Robinson, 1918). This secretion is usually first seen in and around the cumulus, and as the cells of the cumulus loosen the corona radiata becomes more apparent. There is often a sharp line of demarcation between the finely granular secondary secretion and the coarser primary liquor; Dawson and Friedgood (1940) have described in the cat a "streaming" of the secondary liquor into the primary, which breaks up the boundary that separates them.

There is little doubt that the normal maturation changes are induced by pituitary gonadotrophic hormone released in response to the copulation stimulus. Apparently the threshold for maturation is less than that for complete ovulation: Hinsey and Markee (1933a) found that small hypophysectomized rabbits which would not ovulate in response to prolan injection nevertheless underwent maturation to the stage of second polar spindle formation. Furthermore, Pincus and Enzmann (1935) induced maturation in rabbits with as little as one fourth the minimal ovulating dose of gonadotrophic preparation.

Atresia of ovarian follicles may occur at any stage in their development. In typical atresia of small follicles the primary degenerative changes occur in the egg, and these are followed by atrophy of the granulosa. In atresia of medium and large follicles, on the other hand, degeneration of the granulosa is the primary factor, and it may be quite complete before the ovum appears to suffer (Asami, 1920; Pincus and Enzmann, 1937). In typical atresia of large follicles maturation changes in the ovum may follow pronounced degeneration of the granulosa including loosening of the corona. Pincus and Enzmann have suggested that the stimulus to such maturation is some nutritional change in the ovum.

Our histological preparations of the ovaries of rabbits in which ovulation was blocked by drugs include all 4 of the atropine-blocked cases presented in Tables 2 and 3, and 12 of the 15 Dibenamine-blocked cases found in these tables. We also have sections of the ovaries of 7 rabbits which ovulated in spite of Dibenamine and 15 animals whose ovulation was not blocked by atropine.

All four of the atropine-blocked cases gave histological evidence of partial activation of their follicles and, by implication, their hypophyses. The same is true of most of the Dibenamine-blocked cases. This evidence is presented in Fig. 1 to 15 and in the following paragraphs. References are made in Table 3 to the conditions in individual animals and cross-references indicate the appropriate figures in which those conditions are illustrated.

Injection of atropine or Dibenamine into an unmated estrous rabbit produces no follicular activation that is apparent at autopsy, 48 hours later (Figs. 1 and 2). Three of the Dibenamine-blocked animals give little evidence of activation (Fig. 3). Two of the "15-second" Dibenamine and one of the "15-second" atropine animals reveal ova and follicles at 48 hours which are characteristic of the normally stimulated ovary about 4 hours after mating (Figs. 4 and 5). Four other mated and Dibenamine-treated females show fairly typical maturation stages that are characteristic of the normal animal 7 to 10 hours after mating (Figs. 6–9).

The other 3 atropine-blocked rabbits (Table 2) and 9 of the Dibenamine-blocked specimens reveal at 48 hours an atypical or activation-type of follicular atresia. A low-powered photomicrographic comparison between typical and atypical atresias is seen in Figs. 10

and 11. The former, as already mentioned, is characterized in large follicles by a primary degeneration of the granulosa. Atypical or activation-atresia (Figs. 11, 12, 14, 15) is characterized by degeneration of the ovum in an intact follicle; mitotic figures are usually found in the granulosa; secretion of secondary liquor and organization of the corona testify to the activation. The degenerating ova usually stain metachromatically; they may or may not contain vesicular nuclei, and they are often apparently invaded by cellular elements which are stained by aniline blue (Figs. 12 and 14). We have seen similar activation-atresia in follicles which failed to rupture in a normal unblocked ovulation but not in non-stimulated ovaries.

Two experimental animals have revealed typical, though retarded, activation pictures at 48 hours following one treatment and atypical atresia following another. Rabbit #380 showed maturation to polar body formation when atropine was injected at 10 sec. post coitum (Fig. 13), and on another occasion activation-atresia when atropine was administered at 15 sec. post coitum (Fig. 14). Rabbit #383 had atypically atretic follicles following 15 sec. Dibenamine blockage (Fig. 15) and slight typical maturation when atropine was injected at 15 sec. post coitum (Fig. 5).

Histological preparations of follicles which ovulated in spite of 15-second atropine injections (Fig. 16) and 15-second Dibenamine injections (Fig. 17) all showed considerable luteinization at fixation 48 hours post coitum. The Dibenamine-treated specimens appeared to be fully as far advanced in the process as the atropine-injected cases.

*Blocking agents and luteal function.* Ten rabbits were utilized to

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EXPLANATION OF FIGURES ON PLATE 1

Apart from figures 1 and 2, all of the follicles and ova pictured in plates 1 and 2 are from ovaries fixed at laparotomy or autopsy at about 48 hrs. (range 43 to 52 hrs.) after mating with a vasectomized buck.

FIG. 1. Section through a resting graafian follicle of a rabbit ovary fixed at 48 hours after an intravenous injection of Dibenamine. Note the absence both of a corona and of secondary follicular liquor. ×40.

FIG. 2. A higher magnification of the ovum in figure 1, showing the vesicular nature of the cytoplasm, the intact nuclear membrane and a large nucleolus. ×210.

FIG. 3. Section of an ovarian follicle from a rabbit (#372) which received intravenous Dibenamine at 15 seconds post coitum. There is evidence of very slight activation —a little secondary liquor in and around the cumulus and a granulation of the cytoplasm of the ovum. ×60.

FIG. 4. Definite signs of follicular activation including flattening of the nucleus at the periphery of the ovum, corona formation and an observable contrast between the finely granular secondary liquor and the coarser primary liquor. The rabbit (#333) received Dibenamine at 15 seconds post coitum. ×75.

FIG. 5. Ovum from a rabbit (#383) which received atropine at 15 seconds post coitum. The degree of activation is about equal to that in figure 4; under normal conditions this stage would be reached at about 4 hours after copulation. Cf. fig. 15. ×210.

FIG. 6. Evidence of further activation is seen in this follicle from a rabbit (#393) which received Dibenamine at 15 seconds post coitum. There is a sharp boundary, Robinson's "membrane," between primary and secondary liquors, and the spindle for

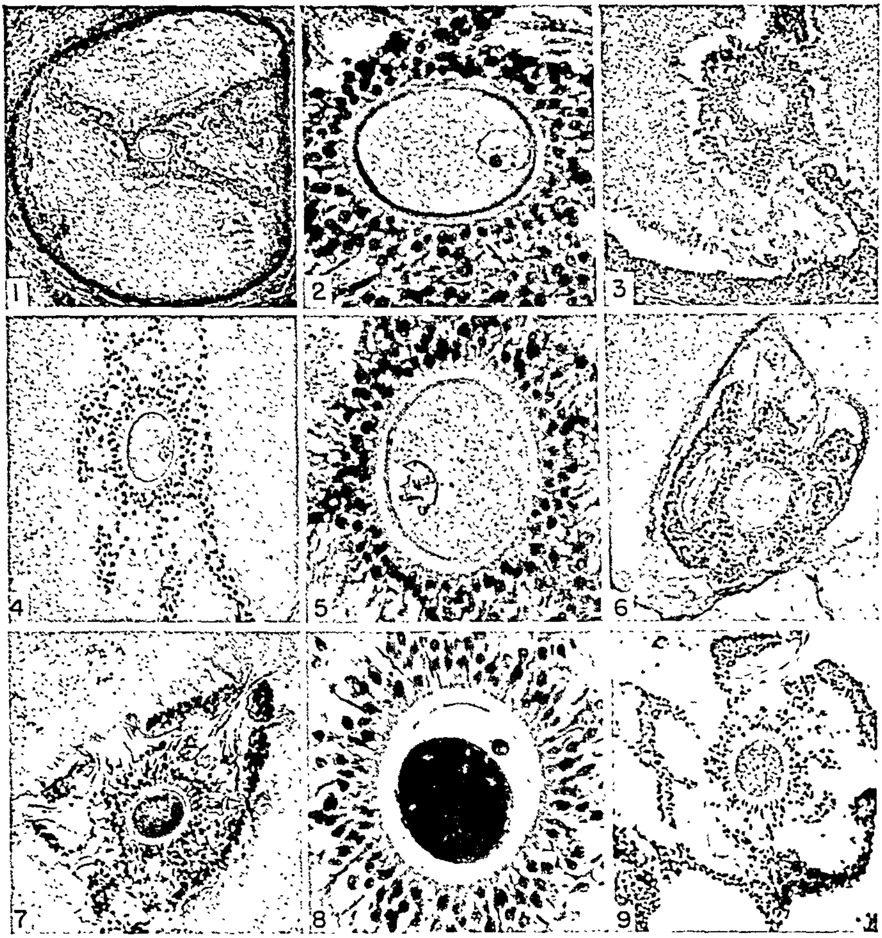

PLATE 1

extrusion of the first polar body is seen as the shadow at the lower left pole of the ovum. ×75.

FIG. 7. Section of a follicle which illustrates "streaming" of the secondary liquor into the primary. The zona pellucida contains a first polar body above the ovum. The rabbit (#379) received Dibenamine at 10 seconds post coitum. The follicle, one of four containing polar bodies in the right ovary of rabbit #379 ,would probably not have ruptured, since the left ovary, fixed eleven days later, contained no recent corpora lutea. ×75.

FIG. 8. Ovum and first polar body from an ovary of rabbit #376, which received Dibenamine at 30 second post coitum. The stage represented by figures 7 and 8 is normally reached at 8 to 10 hours after mating. ×210.

FIG. 9. Section of follicle in which the ovum contains the second polar spindle: the first polar body is not in the plane of section. Note the well developed corona and "streaming" of secondary liquor. It is at this stage of maturation that ovulation ordinarily occurs. The rabbit (#348) received Dibenamine at 15 seconds post coitum. ×75.

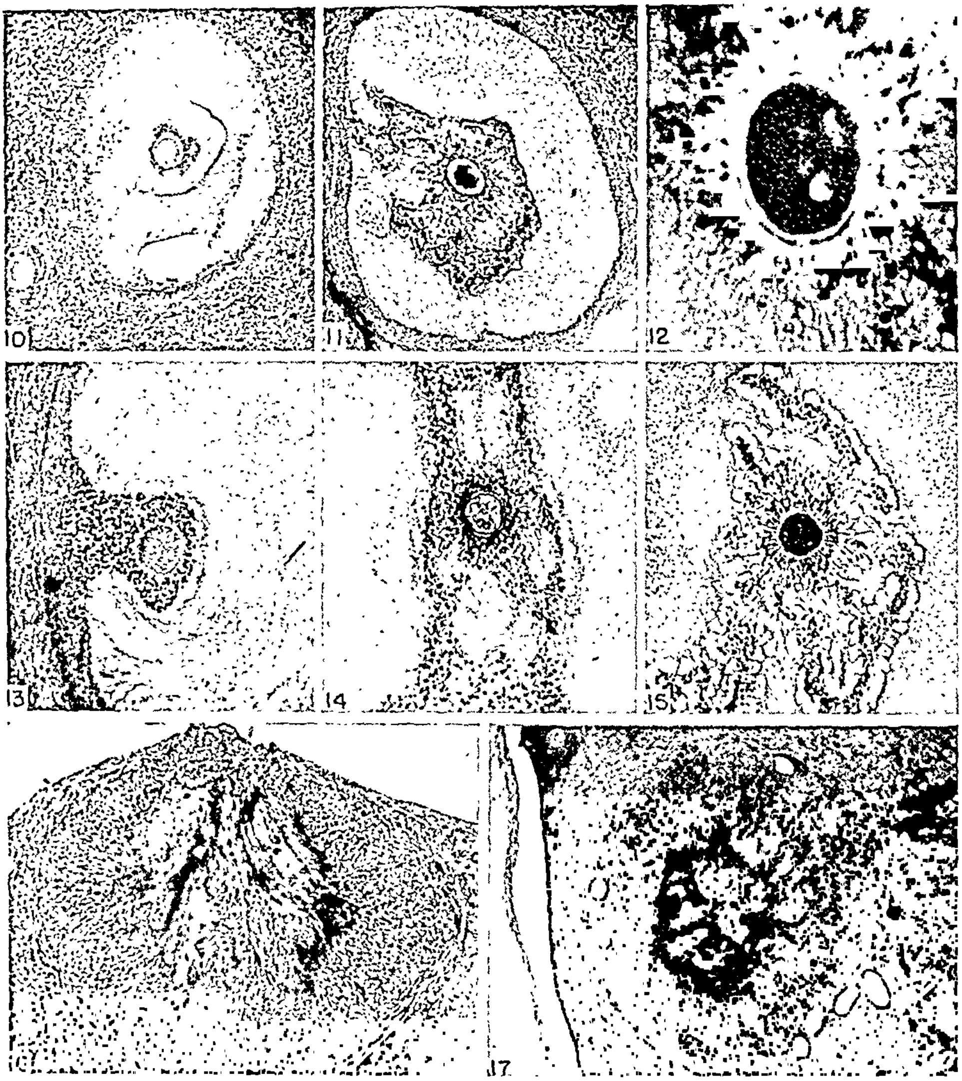


PLATE 2

Explanation of figures

FIG. 10. Large follicle undergoing typical atresia. The granulosa has almost completely degenerated while the ovum, although lacking a vesicular nucleus, is still intact. This follicle (maximum diameter, 0.97 mm.) is only slightly smaller than the resting follicle (maximum diameter, 0.92 mm.) in fig. 1. ×35.

FIG. 11. Section through a follicle undergoing atypical or activation-atresia. The ovum is degenerate while the granulosa is intact. Activation by LH is indicated by the size of the follicle (cf. fig. 10), the definite corona, and the secondary liquor folliculi which is separated from the primary liquor by a distinct "membrane." This follicle and the preceding one were both from an ovary of rabbit #374, which was the only female that failed to ovulate when injected with atropine at 30 seconds post coitum (vide Table 2). ×35.

FIG. 12. Ovum from an atypically atretic follicle. The animal, #384, received Dibenamine at 15 seconds post coitum. Note the distinct corona and the elements (phagocytes?) within the ovum. ×185.

FIG. 13. Section showing ovum with polar body but otherwise slight activation—little secretion of secondary liquor. The follicle was from the right ovary of rabbit #380 after treatment with atropine at 10 seconds post coitum (cf. fig. 14). ×65.

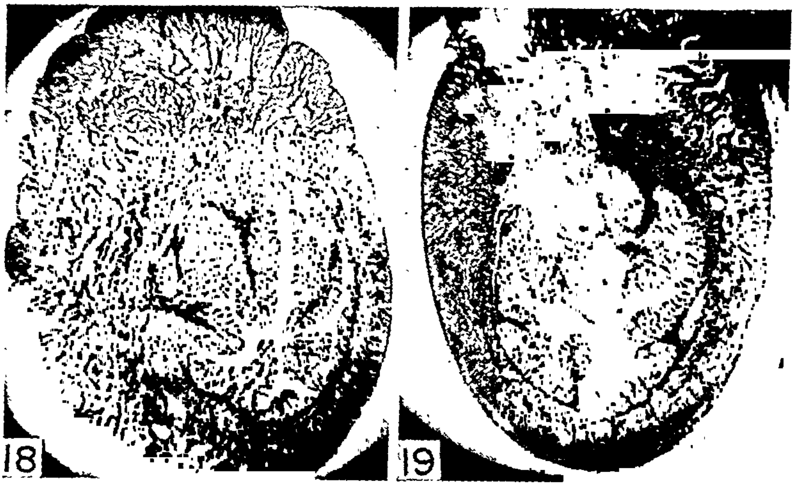


PLATE 3

Explanation of figures

FIG. 18. Cross section of a rabbit uterus fixed 9 days after a sterile mating which was followed within a minute by an intravenous injection of atropine. Note the progestational proliferation of the glands. The corpora lutea looked normal for 9 days of pseudopregnancy. ×8.

FIG. 19. Uterus with deciduomata 11 days after mating and 6 days after threading. Atropine was injected at 5 minutes post coitum and daily thereafter until autopsy. ×8.

determine whether atropine or Dibenamine interfered with the secretion of progesterone. The animals were mated with a vasectomized buck and 6 were injected with atropine and 2 with Dibenamine (30 mg./kg. in each case) at 1 or 5 minutes post coitum; two others served as controls. Two of the 5-minute atropine-treated females also received daily injections of 30 mg. atropine per kg. until the day of autopsy. Laparotomy revealed that all 10 had ovulated. At a second laparotomy 5 days post coitum, the uteri of 7 animals (1 Dibenamine-treated, 4 atropine-treated and 2 controls) were threaded to induce formation of deciduomata. The 3 rabbits whose uteri were not threaded (1 Dibenamine-treated, 2 atropine-treated) were autopsied at 9 days post coitum. Their uteri, in histological sections, all showed progestational proliferation of the glands (Fig. 18). The 7 uterus-

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FIG. 14. Activation-atresia in a follicle of the left ovary of rabbit #380 after atropine injection at 15 seconds post coitum (cf. fig. 13). Definite activation is indicated by the secondary liquor and the corona. The elements within the ovum are perhaps phagocytes; they are probably not blastomeres for a vesicular nucleus is still present in a similarly "invaded" ovum of another follicle in the same ovary. ×65.

FIG. 15. Activation atresia after Dibenamine treatment at 15 seconds post coitum (rabbit #383) for comparison with figure 5. ×65.

FIG. 16. Ruptured follicle at 48 hours after mating. The rabbit (#333) received atropine at 15 seconds post coitum. Cf. figures 4 and 17. ×35.

FIG. 17. Ruptured follicle at 48 hours; Dibenamine at 15 seconds post coitum. The degree of luteinization is approximately equal to that in the atropine-treated case, figure 16. ×35.

threaded animals were autopsied at 5 or 6 days after the threading. Histological preparations revealed an unmistakable deciduoma in at least one uterine horn of each rabbit. The deciduomata were small in two of the atropine-treated females but were extensive in the 2 animals which received the daily atropine injections (Fig. 19). At autopsy all of the corpora lutea looked normal, and histological sections from 2 of the "5-minute" atropine animals showed no apparent morphological changes from the control. Thus atropine and Dibenamine may be said to exert no overt influence on luteal function after the stimulus for LH release has reached the adenohypophysis.

## DISCUSSION

Earlier attempts to block ovulation in the rabbit with atropine differ from the present experiments in two important details: in the former the dosages were much smaller and were administered just prior to copulation. Foster, Haney and Hisaw (1934) reported that atropine in doses up to $2\frac{1}{2}$ mg./kg. injected between 5 and 20 minutes before copulation inhibited ovulation in 7 rabbits. However, Makepeace (1938), repeating their experiments with estrous (post-partum) rabbits, reported that 11 out of 13 ovulated in spite of atropine doses up to 6 mg./kg. Since the experimental conditions in the present work differed so extensively from those in the earlier investigations, the positive results cannot be considered a direct confirmation of the findings of Foster, Haney and Hisaw. The high dosage (30 mg./kg.) employed in the present experiments could not be administered prior to copulation for it threw the animals out of heat for one or more days; Makepeace reported a similar effect in most of the animals injected with 6 mg. atropine per kg. In defense of the possible criticism that our higher dose blocked ovulation by an unspecific debilitating effect on the animals, we need merely point out that a few seconds' delay in injection allowed ovulation to proceed in an unimpaired manner. Actually some of the rabbits in preliminary experiments withstood 50 mg./kg. without noticeable effects beyond pupillary dilation; their sera probably contained high complements of atropinesterase, an enzyme which is found in one out of four rabbits (Glick and Glaubach, 1941). Due to the probable presence of atropinesterase in some of the experimental animals, the 30 mg./kg. dose may have been too weak in certain cases, but the improvement in the percentage of blocking by increasing the rapidity of injection indicates that the time factor may well account for the failures.

Taubenhaus and Soskin (1941) reported that in the rat preliminary treatment of the hypophysis with atropine blocked the pseudopregnancy which usually follows genital stimulation. This they attributed to an inhibition of the release of "luteinizing" hormone but luteotrophic hormone (lactogen) has subsequently been shown to control luteal function in the rat (Astwood, 1941; Evans *et al.*, 1941).

The experiments of Robson (1938) suggest that in the rabbit the hypophyseal control of the secretion of the corpus luteum is an indirect one, but a neural element in such control is indicated by corpus luteum degeneration in rabbits whose pituitary stalks have been cut (Westman and Jacobsohn, 1940). In the present experiments neither atropine nor Dibenamine interfered with luteal function. This could mean, however, merely that single or daily injections of drugs, while capable of blocking the temporary neurogenic stimuli for a rapid surge of gonadotrophic hormone, are of insufficient duration to block chronic neurogenic stimuli as completely as does section of the hypophyseal stalk.

The positive results on cholinergic stimulation of luteotrophic hormone in the rat (Taubenhaus and Soskin, *op. cit.*) and on adrenergic stimulation of LH release in the rabbit (Markee, Sawyer and Hollinshead, 1948) suggest either that various pituitary hormones require different neural mediators or that various species utilize different mediators in their over-all neural control of the adenohypophysis. That the latter, at least in respect to the liberation of LH, is not true has recently been demonstrated by Sawyer, Everett and Markee (1948) and by Everett, Sawyer and Markee (1948), who found that in the rat as in the rabbit the release of LH is blocked by Dibenamine. More recent experiments of the same investigators (unpublished) indicate that the cholinergic-adrenergic mechanism proposed here for the rabbit may also hold true in the stimulation of release of rat LH.

The present work emphasizes the speed with which the coital stimulus reaches the hypophysis. Prior to the appearance of the earlier Dibenamine results (Sawyer, Markee and Hollinshead, 1947) it was thought that the stimulus required up to half an hour to reach the gland. Westman and Jacobsohn (1940) reported that sectioning the stalk as late as 35 minutes post coitum blocks ovulation, and none of their animals whose stalks were cut less than 25 minutes post coitum ovulated. From our more recent knowledge it seems obvious that Westman and Jacobsohn's operation must have interfered with the output of LH rather than the reception of the particular coital stimulus which they were trying to block. Our earlier Dibenamine experiments showed that the coital stimulus reaches the hypophysis in less than 3 minutes. The present Dibenamine results, assuming that a blocking dose reaches the hypophysis within 25 seconds after the start of injection, indicate that in half of the animals sufficient stimulus for the release of an ovulating surge of LH reaches the hypophysis within 55 seconds post coitum. The stimulus for liberation of sufficient LH to activate the follicles of most ovaries and to ovulate some has reached the pituitary within 40 seconds post coitum. The time relationships of the completion of the atropine-blocked component are still less, as some of the "rapid-injection" animals ovu-

lated in spite of a blocking dose which induced pupillary dilation within 25 seconds post coitum.

The neurogenic stimulation of the release of LH is not an all-or-nothing phenomenon since partial activation results in the liberation of reduced amounts of hormone. Such a mechanism probably explains the delayed ovulations observed after artificial stimulation of the hypophysis (Marshall, 1942); some of those ovulations occurred as much as 48 hours after stimulation. In the present work, with laparotomy or autopsy at 48 hours after copulation, no certain cases of delayed ovulation were observed. There was, however, considerable histological evidence of partial activation, and there were many instances of activation combined with atresia of the ovum. Whether this atypical or activation-atresia represents the liberation of more, or less, LH than that required for simple partial maturation, cannot be determined from the present data, which are controversial on that point. The atypically atretic follicles appear to be similar to the cystic follicles produced in the rabbit by electrical stimulation too weak to induce ovulation (Ball and Hartman, 1939). Complete blocking of the release of LH was the exception rather than the rule even in the Dibenamine series, and no cases were observed in the atropine-treated animals. That this inability to block completely the ovulatory mechanism was attributable to the speed of transmission of the copulation stimulus in the rabbit is attested by the recent findings of Everett, Sawyer and Markee (unpublished) in the rat; in that animal the drugs may be administered before the onset of the neurogenic stimulus and complete blockages of the release of LH with either atropine or Dibenamine are common.

A mechanism involving cholinergic and adrenergic components, apparently in sequence, recalls conditions existing in the adrenal medulla and in sympathetic ganglia. In the adrenal medulla Feldberg, Minz and Tzudzimura (1934) demonstrated that adrenaline is released in response to acetylcholine liberated by the splanchnic nerve. We do not believe, however, that systemic adrenaline from the adrenal medulla normally stimulates the release of the hypophyseal LH, for two reasons: (1) No one has been able to induce ovulation in rabbits by the intravascular administration of adrenaline (Markee, Sawyer and Hollinshead, 1948); (2) cutting the pituitary stalk blocks copulation-induced ovulation in the rabbit without removing the major arterial supply (Brooks, 1938), which would import the systemic adrenaline.

In sympathetic ganglia (Dale, 1939) there is considerable evidence that a cholinergic mechanism (liberation of acetylcholine from the preganglionic endings) stimulates an adrenergic mechanism (transmission along the postganglionic fibers and liberation of adrenaline or sympathin from their endings). The cholinergic activity at the ganglion, as also in the adrenal medulla, is a nicotinic action of

acetylcholine, but still probably would be blocked by atropine in dosages as high as 30 mg./kg. (Abdon, 1940). However, it is not probable that the cervical sympathetics are included in the natural route of the coital stimulus, for several workers have removed them without interfering with copulation-induced ovulation (Vogt, 1931, 1933; Hinsey and Markee, 1933b). It is true that Friedgood and Pincus (1935) induced ovulation in the rabbit by stimulating the cervical sympathetic chain electrically, but they employed abnormally strong and prolonged ($\frac{1}{2}$ to $2\frac{1}{2}$ hrs.) stimulation. With stimuli of physiological intensities in the unanaesthetized rabbit, Markee, Sawyer and Hollinshead (1946) were unable to induce ovulation even when the periods of stimulation were prolonged.

Stimulation of the release of LH from the rabbit hypophysis by the injection of adrenaline directly into the gland (Markee, Sawyer and Hollinshead, 1948) has implicated an adrenergic mediator as the final link in neurogenic stimulation. If not from the adrenal medulla or cervical sympathetic nerves, what are its source and pathway? The finding by Westman and Jacobsohn (1937) and Brooks (1938) that rabbits with sectioned pituitary stalks would mate but not ovulate suggested to them that nerve fibers of the stalk transport the neurogenic stimuli which control the hypophysis. Westman and Jacobsohn (1937, 1938) demonstrated a similar importance of the stalk to normal gonadotrophic function in rats, but later Westman, Jacobsohn and Hillarp (1943) eliminated the essentiality of the abundant nerve fibers of the infundibular stalk. They reported numerous nonsympathetic nerve fibers entering the pars distalis through the tuberalis in the rat and also in the rabbit (Hillarp and Jacobsohn, 1943), and that as long as a tuberalis-distalis connection remained intact, gonadotrophic function remained undisturbed. Figure 20A interprets our results in terms of Westman, Jacobsohn and Hillarp's neural pathway with a central cholinergic synapse and a peripheral adrenergic nerve fiber. The central, hypothalamic site for the cholinergic component has a precedent in the recent work of Pickford (1947), who stimulated the release of posterior lobe hormone in the dog by injecting acetylcholine directly into the hypothalamus. Against the nerve fiber localization of the adrenergic component, however, are the following objections: (1) Nerve endings are very scarce in the pars distalis, regardless of source (Rasmussen, 1938, Green and Harris, 1947); (2) Green (1948) has described as reticulum, fibers similar to those diagnosed as nerves by Hillarp and Jacobsohn; (3) Markee, Sawyer and Hollinshead (1946) found the pars distalis practically inexcitable electrically, a condition that is not in keeping with a nerve-ending hypothesis. The finding has been confirmed by Harris (1948) who also reported that the infundibular stalk was electrically inexcitable. Both found that hypothalamic centers, which control the hypophysis, were sensitive to weak electrical stimuli

which were ineffective when applied directly to the adenohypophysis.

The arguments above as well as the findings of Westman, Jacobsohn and Hillarp, that the integrity of the tuberalis-distalis connection is essential for normal gonadotrophic control, may all be used as indirect evidence that the hypophyseal portal system is an important link in the neural control of the anterior pituitary. Wislocki and

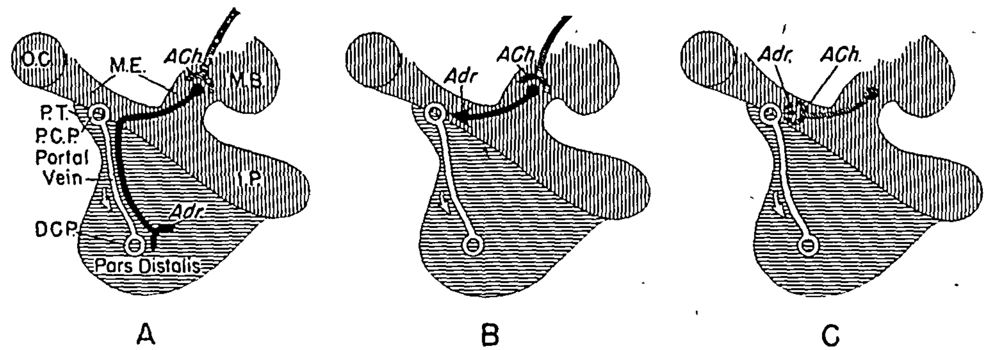


FIG. 20. Diagrammatic sagittal sections of the hypophysis with three possible schemes of the relationships of cholinergic and adrenergic components in the neurogenic stimulus for the release of LH. A. The adrenergic mediator is liberated in the pars distalis from nerve fibers entering via the pars tuberalis and accompanying the portal vessels; the cholinergic mechanism is located at hypothalamic synapses. The cells of the adenohypophysis would thus receive the adrenergic mediator directly from nerve endings. B. The adrenergic mediator is liberated from nerve endings in the median eminence directly into the proximal capillary plexus of the portal system; the cholinergic component has the same locus as in A. C. The adrenergic mediator is liberated into the proximal capillary plexus of the portal system from cells located in the median eminence; the cholinergic mediator is liberated in the median eminence from nerve fibers arising in the hypothalamus. In both B and C the cells of the pars distalis would receive the adrenergic mediator from the hypophyseal portal system at its distal capillary plexus. *ACh.*, source of acetylcholine. *Adr.*, source of adrenergic mediator. *D.C.P.*, distal capillary plexus. I. P., infundibular process. M.B., mamillary body. M.E., median eminence. O.C. optic chiasma. P.C.P., proximal capillary plexus. P.T., pars tuberalis.

King (1936) were the first to suggest that this system carries blood through the tuberal region in the direction of the pars distalis. Their findings have been confirmed and extended by Green and Harris (1947) and by Green (1948). Harris (1948) has recently summarized much of the evidence in favor of a neurovascular control of the adenohypophysis. Harris' suggestion that the portal system has rapid powers of regeneration may well account for the lack of impairment of gonadotrophic control following stalk section in the guinea pig (Dempsey, 1939) and in the rat (Dempsey and Uotila, 1940). Brolin (1947) has recently emphasized the importance of the tuberalis-distalis connection in the control of thyrotrophic hormone.

Figure 20, B and C, give two possible interpretations of the loci of the cholinergic and adrenergic components of the neural control with the portal system as the final pathway in each. Figure 20, B, has the cholinergic component at a central synapse in the hypothalamus and

the adrenergic agent liberated from nerve endings related to the proximal capillary plexus in the median eminence. An anatomical basis for the latter in the rabbit is found in Green and Harris' (1947) description of nerve endings in the walls of the looped and tufted vessels of the proximal capillary plexus.

Figure 20, C, places both cholinergic and adrenergic mechanisms peripherally, with acetylcholine from nerve endings stimulating the release of the adrenergic mediator from elements in the median eminence. The term "peripherally" is used advisedly for the median eminence is no longer considered part of the hypothalamus but of the posterior lobe of the hypophysis (Rioch, Wislocki and O'Leary, 1940). There is as yet no morphological basis for the location of the adrenergic component in such an hypothesis, but our work provides indirect physiological evidence for it. The fact that atropine is effective at all, when injected intravenously post coitum, suggests a peripheral locus of action for the cholinergic mediator. One might expect central neural synapses to be activated within a matter of milliseconds, whereas the injection of atropine and circulation time consume many seconds. The differences between effective atropine and Dibenamine injection times, again of the order of several seconds, implies that stimulation of the liberation of an effective amount of adrenergic mediator is also a slow process. Such time relationships would fit the liberation of adrenaline from cells around the proximal capillary plexus as well, at least, as from nerve endings. The observation of O'Connor (1947) and Green (1948), that the median eminence contains many pituicytes yet produces very little posterior lobe hormone, may have significance in this connection. Whether produced by nerve endings or cells, adrenaline is sufficiently stable to withstand inactivation during a short vascular journey, which is a characteristic probably not shared by acetylcholine in the absence of anticholinesterases.

## SUMMARY AND CONCLUSIONS

Earlier work has demonstrated that an adrenergic mediator is involved in the neurogenic control of the release of luteinizing hormone from the rabbit hypophysis. Ovulation, which signifies the release of luteinizing hormone, was induced by instilling adrenaline, but not acetylcholine, directly into the anterior hypophysis, and copulation-induced ovulation was blocked by the rapid post-coital injection of the adrenolytic drug Dibenamine.

Like Dibenamine, atropine sulfate blocks ovulation when injected very quickly after copulation. However, to be equally effective atropine must be given earlier than Dibenamine: when injection was started at 15 seconds after the termination of copulation most of the atropine-treated rabbits ovulated, whereas most of the Dibenamine-injected animals failed to ovulate. Thus, a cholinergic (atropine-blocked) component of the copulation-initiated neurogenic stimulus

appears to precede the well established adrenergic component.

The ovarian follicles whose rupture has been blocked by atropine or Dibenamine almost invariably give histological evidence of partial activation. This includes characteristic preovulatory changes and also an atypical atresia of large follicles, in which the ova precede the granulosa in degeneration. After a follicle has ruptured, the normal development and function of the corpus luteum does not appear to be affected by atropine or Dibenamine.

The natural neurogenic stimulus at copulation traverses the pituitary stalk, and the final pathway, distal to the median eminence, is probably the hypophyseal portal system. Possibly in this system, as in the adrenal medulla and sympathetic ganglia, the cholinergic component stimulates the secretion of the adrenergic mediator, which in turn stimulates the hypophyseal cells to release luteinizing hormone.

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# THE RESPONSE OF THE THYROID GLAND IN NORMAL HUMAN SUBJECTS TO THE ADMINISTRATION OF THYROTROPIN, AS SHOWN BY STUDIES WITH $I^{131}$*

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THE actions of thyrotropin in various laboratory animals have been studied in considerable detail. Following its administration the thyroid gland becomes hypertrophied and hyperplastic, the content of protein-bound iodine decreases, but there is an increase in the inorganic iodine fraction. The circulating protein-bound iodine and the basal metabolic rate are elevated. An increased capacity of the gland to fix iodine has been demonstrated by the use of $I^{131}$.

Since none of these methods has been generally applicable to the study of normal human beings, and since relatively pure preparations of the hormone have not been readily available, little information has accumulated regarding the actions of thyrotropin in man.

In the present study radioiodine methods originally designed for demonstrating the action of thyroid inhibiting substances were adapted to study the influence of single doses of thyrotropin on the several phases of iodine metabolism in the normal human thyroid gland.

## METHODS

The methods used were those which have previously been described (Stanley and Astwood 1947, 1948). The total iodine accumulation was studied by administering to each subject by mouth a tracer dose of $I^{131}$, diluted in normal saline solution, without added carrier. The collection of radioiodine by the thyroid gland was detected by serial counting at thirty- to sixty-minute intervals with a shielded Geiger-Müller counter in contact with the skin over the thyroid gland above the sternal notch. The resulting data, expressed as counts per second per 100 microcuries of administered $I^{131}$, were plotted versus the square root of the time in minutes, thus converting the parabolic uptake curve to a straight line. The slope of this straight line, referred to as the accumulation gradient, served as an index of the rate of iodine uptake. Measurements during the first one to two hours of the study

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allowed a fairly accurate prediction of the course of uptake for the remainder of the period; deviations from this course could be related to the effects of administered inhibiting or stimulating substances (Figure 1).

This approximate straight-line relationship was found to hold for eight hours or more in normal subjects, but the uptake was virtually completed

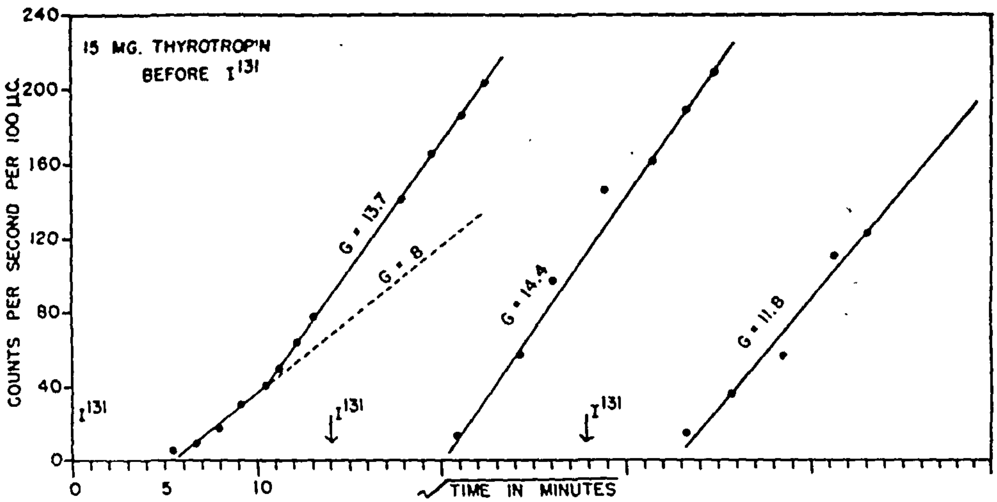


FIG. 1. The influence of thyrotropin on the accumulation of radioiodine in a normal young woman. Fifteen milligrams of thyrotropin was administered intravenously five hours and forty-one minutes before the $I^{131}$ was taken by mouth. During the two hours following the tracer dose the course of uptake was established with an accumulation gradient of 8. At two hours and six minutes after the $I^{131}$, or seven hours and forty-seven minutes after thyrotropin was given, the rate of accumulation increased to a gradient of 13.7. On the second day the uptake following a second tracer was higher (accumulation gradient—14.4), but was decreasing on the third day.

by the end of twenty-four hours in these and in the group given thyrotropin· Very little change occurred for several days thereafter. Consequently, it was possible to make a second tracer study as early as twenty-four hours after the first.

The uptake of iodide ion was measured after the administration of mercaptoimidazole, an antithyroid drug of the thiouracil type, in quantities sufficient to inhibit as completely as possible the organic binding of iodine in the thyroid. Usually 100 milligrams of mercaptoimidazole were given one to two hours before the $I^{131}$ was given, followed by 50 milligrams every eight hours for the duration of the experiment. It could be readily demonstrated that the radioactive iodine entrapped was *iodide* and that organic binding did not occur to an appreciable extent except in the instances mentioned below. The administration of 1 to 2 grams of potassium thiocyanate caused a rapid discharge of the thyroid iodide and permitted identification of that portion of the total iodine which was in ionic form (Figure 2). In plotting these results, an ordinary linear time scale was adequate for the abscissa. In some instances the logarithm of the thyroid iodide content was plotted against time. Since the iodide in the thyroid was in a state of equilibrium with the blood iodide, they both decreased in a logarithmic fashion as the blood level was reduced by renal excretion.

In order to estimate the iodide-concentrating capacity, it was necessary

to measure quantitatively the radioactivity in the thyroid gland as accurately as possible. The best approximation that could be obtained was calculated from determinations made at a relatively large distance (26.5 cm.) from the thyroid using a sensitive gamma counter. In view of the fact that it was not possible to shield completely the radiation from other body tissues,

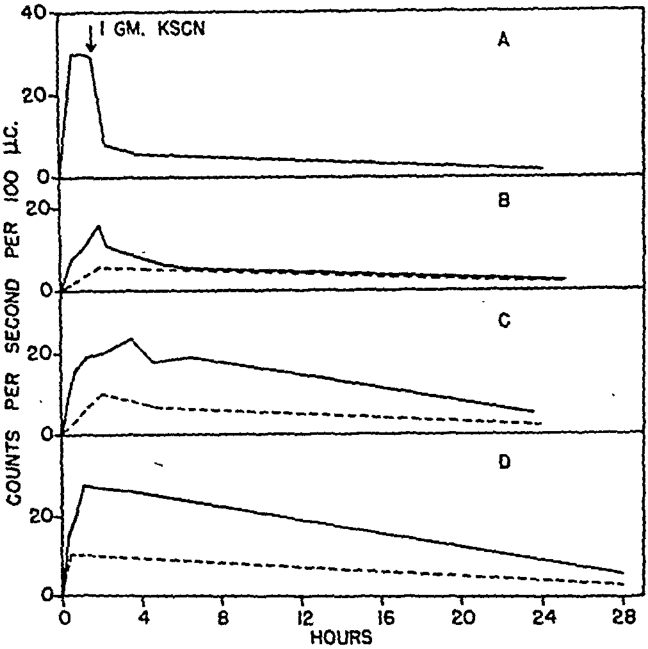


FIG. 2. The accumulation and subsequent fate of radioactive iodide ion in the thyroid glands of four normal persons is shown by solid lines. For comparison, the radioactivity over the left anterior chest are indicated by dotted lines. In all cases the administration of an effective dose of mercaptoimidazole preceded the dose of tracer by about an hour. Proof that the tracer was in the form of the iodide ion was provided by the rapid discharge from the gland of virtually all the contained radioactivity following administration of 1 gram of potassium thiocyanate in subject A. In those individuals in whom complete inhibition of organic binding was maintained, and who were not given thiocyanate, there was a gradual loss of radioiodide from the gland during twenty-four to forty-eight hours. Compare with Fig. 4.

and because the factor of scattering could not be accurately estimated, the determination of the total radioactive iodine in the thyroid gland was possibly in error by 20 to 25 per cent. In order to calculate the quantity of radioactive iodide in 1 gram of thyroid tissue, the weight of the gland was assumed to be 25 grams in each subject. This estimate might also have been in error by 50 to 100 per cent. However, it was thought that any errors which occurred were constant in the same subject, so that the comparative determinations were quite accurate.

In some experiments additional tracer doses were given at intervals of one or two days. The rates of accumulation of these additional tracers were calculated in such a manner that the residual radioactivity from former doses was subtracted from the subsequent readings. Thus, the plotted figures show only those increments of the total radioactivity in the gland produced

by the dose of $I^{131}$ administered for the respective test. In most such multiple-dose experiments each dose of $I^{131}$ was 20 to 50 microcuries, depending upon the rate of uptake expected, but all values were expressed in terms of 100 microcuries.

The thyrotropin used[2] was freshly dissolved in 1 to 3 milliliters of distilled water or normal saline and administered as a single dose intramuscularly, or intravenously.

Two groups of subjects of both sexes were given thyrotropin during the study. The first was composed of seventeen individuals who had no abnormalities of thyroid or pituitary function, and who had been taking no drugs known to influence these glands. Patients who were taking thyroid in varying amounts made up the second group of six people. On the basis of history these latter patients were considered to have had normal thyroid function before they were given desiccated thyroid; this opinion was substantiated by the course of events following the discontinuation of the medication. Numerous additional subjects of both types received no thyrotropin and served as controls.

## RESULTS

*Total Iodine Accumulation in Normal Subjects:* For purposes of comparison, the findings in 265 normal subjects tested for various purposes during the past two years were summarized. The majority of subjects were between fifteen and fifty years of age, and most were females. The accumulation gradients varied between 2 and 13 in 96.5 per cent of instances. The ten subjects whose gradients fell outside these limits had higher values. Eight of these unusually high values were observed during the early work with radioiodine; there is reason to believe that due to a difference in methods of standardization the size of the tracer dose was about 50 per cent higher than it should have been.

Since the data to be presented were derived in many instances from studies with multiple doses of tracer radioiodine, the constancy of the accumulation gradient in repeated tests on untreated control patients was investigated. Thirty-six subjects were studied more than once. When several months elapsed between the tests, rather wide variations were encountered in some instances. Consequently an additional group of four normal subjects was tested on successive or alternate days. As shown in Table 1, the rates of uptake on repeated tests were quite uniform. The close agreement in these results indicated that this method could be used to study changes in thyroid activity over the course of several days.

*Effects of Thyroid Medication:* The normal subjects who were receiving 4 to 8 grains of thyroid daily exhibited a complete or very

[2] The thyrotropin was generously supplied through the kindness of Dr. John R. Mote of the Armour Laboratories, Chicago, Illinois. It was a purified preparation from beef anterior pituitary glands with a stated potency of 10 to 20 Waleszek-Koch chick units per milligram. This unit is defined as the total amount which, when injected twice daily for three days, will reduce the thyroid iodine of the chick by 50 per cent.

nearly complete suppression of iodine uptake (Figure 3). The suppressive effect of chronic treatment with smaller doses of desiccated thyroid has not been studied quantitatively, but from the few tests which were made it was evident that there was considerable individual variation. In one subject 1½ grains daily produced nearly complete inhibition which continued for several weeks after treatment was stopped. While 3 grains daily in one individual was sufficient to produce only a moderate inhibition, in another given this dose the suppression was complete.

TABLE 1. THE ACCUMULATION GRADIENTS CALCULATED ON SUCCESSIVE OR ALTERNATE DAYS IN FOUR NORMAL SUBJECTS

| Days | Gradient | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Subject A | 4.2 | 4.2 | 3.7 | | |
| Subject B | 9.3 | | 9.0 | | 9.1 |
| Subject C | 6.3 | | 6.4 | | 6.2 |
| Subject D | 10.1 | 10.0 | 10.2 | | |

*Influence of Thyrotropin:* The effect of thyrotropin on the *total iodine accumulation* by the thyroid was studied in fifteen patients who were considered to have normal or potentially normal thyroid function before suppression by desiccated thyroid.

Examples of the type of response which was observed are shown in Figures 1 and 3. Two individuals received single doses of $I^{131}$ in conjunction with the thyrotropin, while the others were given repeated tracer doses. After a tracer dose of radioiodine, the course of uptake by the thyroid region followed a distinctive pattern during the first eight to twelve hours, and changes in the rate of uptake were easily detected during this interval. When 15 or 30 milligrams of thyrotropin were given by intravenous or intramuscular injection during the course of the accumulation of a tracer dose, no effect was observed during the ensuing six or seven hours (Figure 3, C). Subsequent tests were, therefore, made by administering the thyrotropin *before* the tracer study was begun. Two subjects studied in this way exhibited an abrupt increase in the accumulation gradients at about eight hours after the injection of thyrotropin (Figure 1). In others the increase was more gradual; it was just detectable at eight hours (Figure 3, A), and a more rapid acceleration ensued thirteen to fourteen hours following the injection (Figure 3, A and B).

During the second twenty-four-hour period following the injection of 15 milligrams, stimulation of iodine accumulation was at its height. During the third day, there was a decline toward the preinjection value. The subjects who continued taking thyroid and whose initial accumulation gradients approached zero exhibited very low rates of uptake after the fourth day. This indicated that the stimulat-

ing effect of the hormone had completely subsided by the end of the fourth day following injection (Figure 3, A). After the administration of 30 milligrams, the stimulating action was still demonstrable for five days (Figure 3 C).

In one patient (Figure 3, B), whose dose of 3 grains daily of desic-

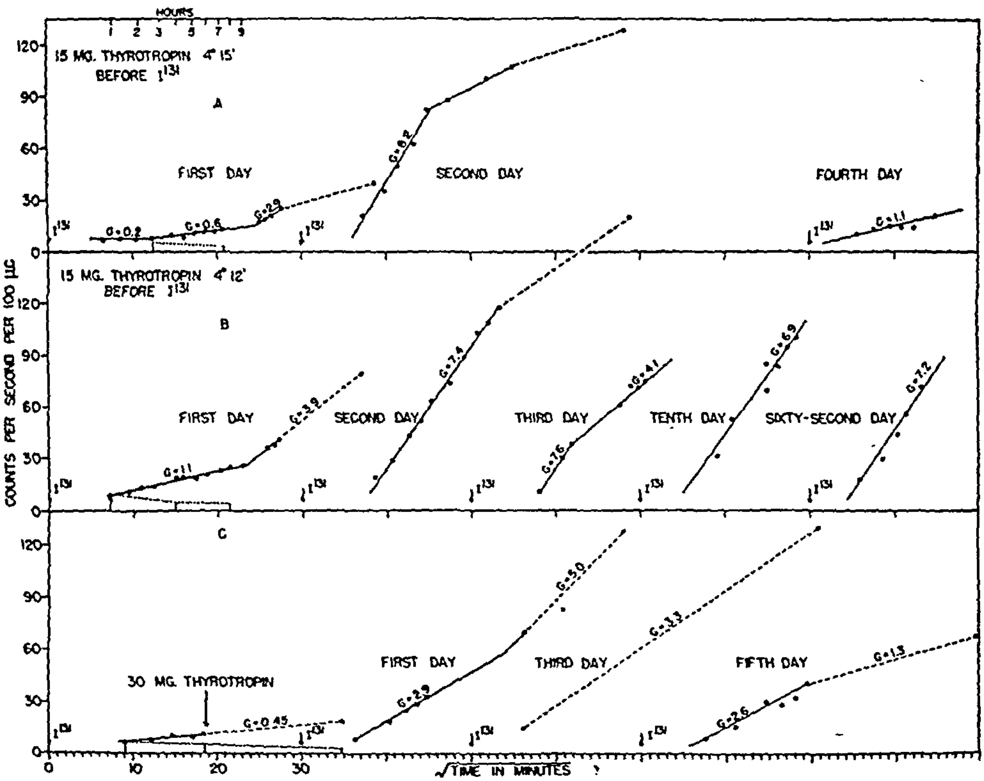


FIG. 3. The influence of thyrotropin on the iodine uptake in three normal persons who had been taking thyroid. Patient A had taken 5 grains daily, B, 3 grains daily, and C, 6 grains daily. The medication of subjects A and C was continued throughout the study, but in B the thyroid was discontinued ten days before the experiment. Each patient received a single dose of thyrotropin at the time indicated. In patient A an increased rate of uptake began eight hours after injection of the thyrotropin. This became more pronounced at fourteen hours and was maximal on the second day. On the fourth day the effect had nearly completely subsided. The sequence of changes observed in subject C was similar, but the effects of the thyrotropin were still evident on the fifth day. In B, the uptake continued essentially unchanged after the third day, presumably under the influence of endogenous thyrotropin.

cated thyroid had been discontinued ten days prior to the first test, a different sequence was observed. The control uptake was low, but not zero, indicating that function was not completely suppressed. The maximal augmentation of uptake occurred on the second day following the injection of 15 milligrams of thyrotropin, and the increased rate of accumulation was only slightly diminished on the third day. One week later, with no treatment of any sort in the meanwhile, the iodine uptake was again at the high level of the second day. On the sixty-second day after the initial injection thyroid function was still

normal without further treatment. Presumably a normal thyroid-pituitary relationship was re-established at about the time the exogenous thyrotropin was administered.

*The Iodide Uptake and the Thyroid-to-Blood-Iodide Ratio* were studied in eight normal subjects who had not been given thyroid. Inhibition of organic binding of iodine in the thyroid gland was continuously maintained with mercaptoimidazole throughout each series of tests. The influence of thyrotropin on the thyroid iodide content was observed over the course of twenty-four hours or longer.

When organic binding of iodine is prevented, a tracer dose of $I^{131}$ labels the iodide ion in the thyroid and in the body as a whole. Equilibrium is quickly established so that one to three hours after the tracer is given the radioiodide in the thyroid region is at a maximum. Thereafter the thyroid radioiodide gradually declines as the body's $I^{131}$ is slowly lost in the urine and replaced by $I^{127}$ from endogenous and exogenous sources (Figures 2 and 5 A, B).

When thyrotropin was given, the rate of decline in the thyroid's radiodine was markedly modified; there was either much less of a decrease, no decline, or as much as a 90 per cent increase in the iodide levels one day later (Figure 4). To gain further information about the magnitude of the increased capability of the thyroid for concentrating iodide, the amounts of radioactivity in the serum were correlated with the thyroid radioiodide determined at intervals before and following the action of thyrotropin. When no thyrotropin was given, it was observed that the ratio of the iodide in the thyroid to the iodide in the serum remained nearly constant for one to two days (Figure 5, A and B). In the instance of subject A shown in Figure 5, the concentration of iodine in the thyroid during the second day was somewhat higher than expected, and it is possible that this was caused by the organic binding of a small amount of iodine. After the administration of thyrotropin, the ratio was constant for approximately eight hours and then increased markedly. In one experiment the change was progressive until the thirty-first hour, after which it remained approximately constant for fifteen hours (Figure 5, C). The capacity of the thyroid for concentrating iodide was increased to 6.5 times the control value in this patient.

Other subjects were given multiple tracer doses to study the influence of single injections of thyrotropin on the iodide uptake. In one patient (Figure 4, C) the course during the first 23½ hours after 30 milligrams of thyrotropin was as described above; between 7½ hours and 23½ hours there was a rise in radioactivity in the gland even though the radioactivity in the body as a whole had fallen markedly. The greatly increased iodide-concentrating capacity was then studied by a second tracer dose which revealed a maximal concentration eight times that of the first day. This high concentration diminished unusually quickly during the balance of the second twenty-four-hour pe-

riod at the termination of which the radioactive iodide level from the second tracer was less than it was at the end of the first twenty-four-hour period. At the beginning of the third day the maximal accumulation from a third tracer dose, forty-eight hours after the thyrotropin, was slightly less than three times the control value.

In a second subject (Figure 4, B) studied for 42½ hours after a single tracer dose, the usual gradual decline in radioactive iodide did

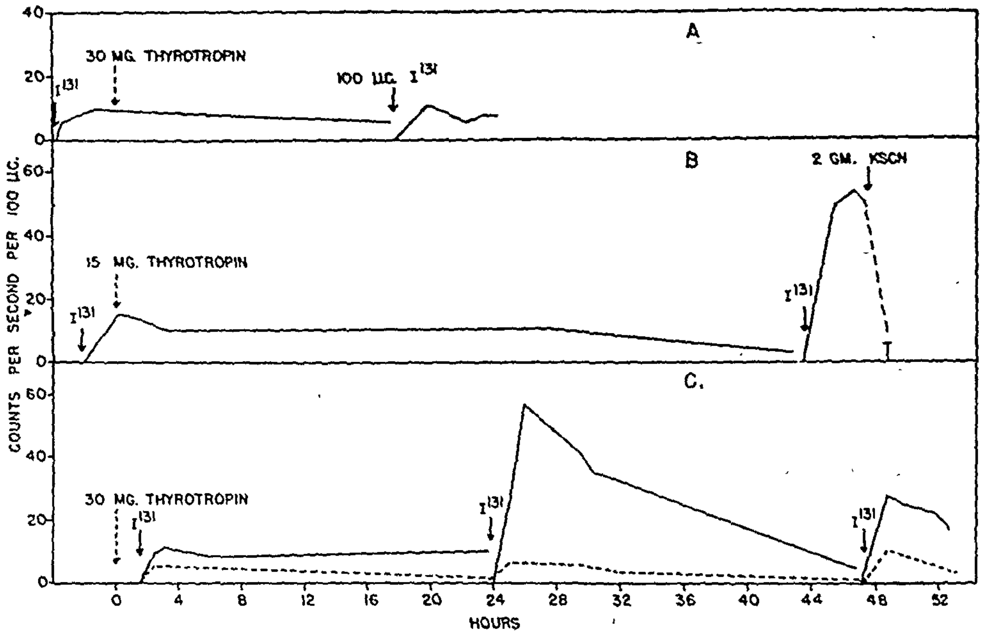


FIG. 4. Tracer studies on the iodide content of the thyroid in the three normal subjects above were performed in the same way as those shown in Figure 2, except for the injection of thyrotropin. During the twenty-four hours following the administration of thyrotropin the expected decrease in radioactivity in the thyroid was partially prevented, and in B and C there was actually a slight increase during this period. Twenty-four to forty-eight hours after the injection of thyrotropin there was an increased capacity for the concentration of iodide. In B, the nearly complete loss of radioactivity after thiocyanate showed that the increased accumulation was actually iodide ion and not organically bound iodine.

not occur until thirty hours after the thyrotropin was given. A second tracer revealed that the iodide content of the thyroid gland was 3.4 times higher than it was before the thyrotropin effect began. At the end of the test the prompt discharge of 94 per cent of the radioactivity from the gland after 2 grams of potassium thiocyanate indicated that virtually all of the iodine was still in the form of iodide, and that only a negligible degree of organic binding had occurred.

*Effect of Thyrotropin on the Discharge of Stored Thyroid Hormone:* Following injections of thyrotropin in the chick, Keating *et al.* showed a rapid loss of stored hormone from the thyroid; this occurred during the first twenty-four hours, while a significant increase in iodine ac-

cumulation did not take place until the second day. This suggested that an emptying of the follicles of their contents was a prerequisite for an increased iodine uptake.

It was of interest to determine whether a single injection of thyrotropin would result in a measurable loss of stored hormone. In the subjects who were given 15 or 30 milligrams of thyrotropin, the maximal accumulations attained during the first twenty-four hours after the tracer were not followed by significant declines during the following two to four days. Two other subjects were first given a tracer dose of $I^{131}$ and then, when the uptake had ceased, were given

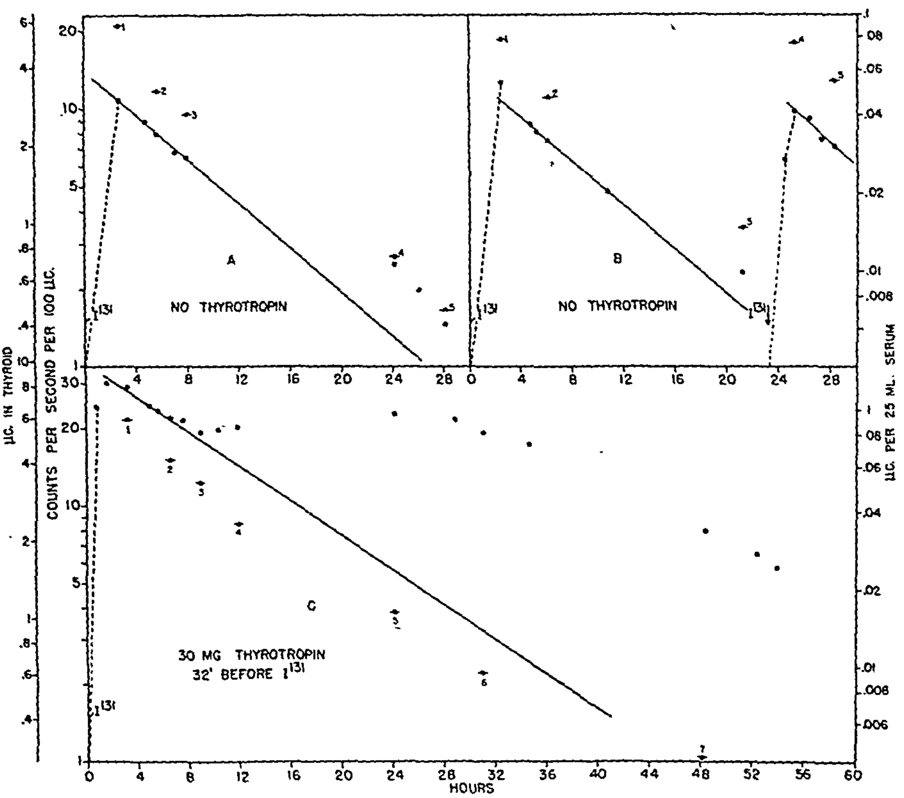


FIG. 5. The influence of thyrotropin on the capacity of the normal thyroid gland to concentrate iodide ion. Each of the three normal subjects above received repeated doses of mercaptoimidazole to inhibit the organic binding of iodine. The solid circles show the radioactivity of the thyroid region, and the crossed circles indicate the radioactivity in the serum. The actual thyroid:serum iodide ratios were:

A. (1) 34, (2) 43, (3) 43.2, (4) *59.9*, (5) *78.1*
B. (1) 43.9, (2) 43.3, (3) 42.8, (4) 34.6, (5) 35.2
C. (1) 85.5, (2) 92.5, (3) 99.5, (4) 153, (5) 378, (6) 554, (7) 494, (8) 543.

It will be seen in (A) that the last two ratios are higher than the first three. This is due to a higher than expected thyroid concentration, probably caused by the organic binding of a small amount of iodine. In subject C under the influence of thyrotropin there was a marked increase in the ratio beginning at about eight hours after injection and attaining a value of 6.47 times the control values at thirty-one hours.

a single dose of 30 milligrams of thyrotropin. Serial determinations extending over thirteen hours and seven days, respectively, revealed no significant loss of the stored $I^{131}$.

In view of the large store of hormone contained in the normal human thyroid, it was perhaps to be expected that no loss of the labeled organic iodine would be detectable following such a brief period of stimulation.

## DISCUSSION

The latent period of eight or more hours between the time of thyrotropin administration and the first detectable increase in thyroid activity is difficult to understand. Numerous experiments with various anterior pituitary hormones have shown that frequent injections are necessary to achieve maximal effects. Most anterior pituitary hormones are far more effective if the daily dose is subdivided and given every few hours, suggesting that hormones of this type are rapidly eliminated from the circulation. It would seem likely that the maximal stimulus to the thyroid gland following a single injection of thyrotropin would occur during the first hour or so and that by eight hours, when the first detectable increase in function is noted, the quantity of circulating thyrotropin would be already markedly decreased. Certainly it would be reasonable to suppose that by the second or third day, when evidences of stimulation are still clearly manifest, the injected thyrotropin has largely been eliminated. It would seem as if thyrotropin in its action upon the thyroid cell merely initiates a sequence of changes which then proceeds without its further influence.

It was of interest to find that the two aspects of thyroid function studied were influenced by thyrotropin in a similar manner. The increased organic binding of iodine was first detectable eight hours after the thyrotropin was given and became maximal some hours later. As far as could be determined, the increased capacity of the thyroid to concentrate iodide ion exhibited identical time relationships. As these two aspects of thyroid activity were stimulated simultaneously, it would not be reasonable to assume that one was primary and the other secondary. Rather it would appear that these two processes merely reflected an increased activity on the part of the thyroid cell induced by the injected thyrotropin. When the capacity of the cell to bind iodine was increased, an increased quantity of iodide ion was at the same time provided; when the cell was caused to concentrate more iodide, it at the same time could convert it more rapidly into an organic form. Inasmuch as the increase in iodide-concentrating capacity occurred when organic binding was inhibited, the binding process was obviously unessential for the phenomenon of augmentation of iodide concentration. It could not, however, be stated that the increase in iodide concentration did not facilitate a more rapid binding

of this iodine, but various considerations suggested that this would not be a large factor.

It would be of interest to know whether the increased rate of secretion of thyroid hormone follows the same temporal pattern after thyrotropin as the other two aspects of thyroid activity which have been studied here. From studies of radioautographs Leblond and Gross have concluded that all of the functions of the thyroid cell proceed simultaneously. They have pointed out that, in analogy to the intestinal mucosa, the thyroid epithelium could easily secrete a protein hormone into the follicle at the same time as it digested and secreted the stored hormone into the blood. If this concept is correct, it would suggest that thyrotropin stimulates the several components of thyroid function simultaneously; it enhances the capacity of the thyroid cell to concentrate iodide ion, to bind iodine to protein, to secrete thyroid protein into the follicle, to digest stored hormone, and to secrete it into the blood stream.

From the previous studies on the iodide-concentrating capacity of the thyroid gland (Stanley and Astwood, 1948), it was suggested that an increased ability of the thyroid to concentrate iodide ion correlated in some way with hypertrophy and hyperplasia. The experiments reported in the present communication suggested that an increased capacity for iodide was a reflection of the action of thyrotropin on thyroid tissue. The ratio of the concentration of iodide ion in thyroid tissue to that in the serum was found to vary between 34 and 94, and averaged 50, in three normal persons in the present study. The means by which this estimate was made was subject to a considerable margin of error, but it was of interest that the ratio was of the same magnitude as that found by VanderLaan and VanderLaan (1947) in normal rats. Here a more exact measurement was possible and the ratio averaged 25. Likewise, the high value of 554 shown by one patient given thyrotropin might be compared with the mean of 240 found in rats whose glands were made hyperplastic by the administration of propylthiouracil.

The studies reported above have practical implications. One is able to determine whether or not a patient being treated with thyroid does or does not have a potentially functional thyroid gland. It is common clinical experience that when euthyroid patients are treated with thyroid, the withdrawal of medication is sometimes followed by transient symptoms and signs of hypothyroidism. This phenomenon has been carefully studied by Riggs, Man, and Winkler, who showed that when large doses of thyroid were discontinued the metabolic rate, serum cholesterol level, and the concentration of serum-precipitable iodine changed from values in the range of mild hyperthyroidism to those of myxedema. It was only after four to six weeks that these criteria of thyroid function reverted to normal. The use of thyrotropin in conjunction with radioactive iodine makes it possible to

determine quickly whether the thyroid gland of the thyroid-treated patient is present and normally responsive.


## SUMMARY

Studies with $I^{131}$ of the effects of a single injection of 15 or 30 milligrams of thyrotropin in 23 normal subjects have been made. For eight hours after the injection no effect on the rate of iodine uptake by the thyroid could be detected, but thereafter a marked acceleration, becoming maximum in twenty-four to forty-eight hours, was observed. The rate of turnover of iodine returned to the pre-injection level in four to five days. This sequence of events was still more clearly exhibited by individuals whose thyroids had been compensatorily suppressed by thyroid medication. When the organic binding of iodine was prevented by the use of mercaptoimdazole, it could be shown that the iodide-concentrating capacity of the thyroid was similarly increased by thyrotropin and the time relationships of this increase were the same as those of the total iodine accumulation. Following a single injection of thyrotropin, it was not possible to detect any loss of stored thyroid hormone.


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# THE TRANSPORT OF PROGESTERONE IN BLOOD[1]

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THIS study was undertaken with the objective of determining the order of concentration of progesterone in the blood of certain representative mammalian species and of ascertaining the form in which this hormone is transported in the blood. It seems generally supposed that steroid hormones in the blood are intimately associated with protein (Edsall, 1947), although evidence for this relationship has, so far as we are aware, been presented only for estrogen (Häussler, 1936; Mühlbock, 1939; Rakoff, Paschkis, and Cantarow, 1943; Szego and Roberts, 1946, 1947; Roberts and Szego, 1946; Boettiger, 1946).

## METHODS

No effort was made to examine any reproductive state; the only consideration was to be reasonably confident that the blood to be examined would contain progesterone. Accordingly, blood was obtained from pseudopregnant rabbits (virgins, six or seven days after injection of gonadotrophin), a rhesus monkey in the luteal phase of the menstrual cycle, a woman considered to be in the eighth week of pregnancy, and a group of mice five days after mating. Examination revealed that not more than one-half of the donor mice were pregnant. The blood from the woman and the monkey was drawn from a superficial vein; that from the rabbits and mice was obtained by cardiac puncture. Sodium citrate was employed as the anticoagulant.

The assays of progesterone were done by the intrauterine injection method in mice (Hooker and Forbes, 1947), which detects 0.0002 μg. of progesterone in a standard volume of 0.0006 ml. of vehicle, a concentration of 0.33 μg. per ml. The sensitivity of the test is the same whether sesame oil or 0.9 per cent saline is the vehicle (Forbes and Hooker, 1948). The concentration of progesterone was ascertained by injecting serial dilutions of the specimen to be tested, the greatest dilution giving a positive test being considered to reflect the concentration. For example, if a plasma sample gave a positive test when diluted 18 times and a negative test when diluted 20 times, the concentration is recorded as 6.0 μg. per ml. ($18 \times 0.33$). It will be apparent that the precision of the value depended upon the gradations in the dilutions tested.

The values for raw whole blood and for raw plasma were obtained by testing serial dilutions of each made with 0.9 per cent saline. Separation of

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the free and bound progesterone in plasma was attempted in two ways. In the first the sample was extracted with ether, a procedure used by Rakoff, Paschkis, and Cantarow (1943) to obtain the free estrogen in blood. A measured, small amount of sesame oil was added to the ethereal extract, and the ether was then removed by distillation under diminished pressure, leaving the material extracted by the ether in solution in the oil. This fraction was labelled "free" progesterone. Following extraction with ether the plasma residue was submitted to hydrolysis by bringing it to pH 0.5–1.0 with concentrated hydrochloric acid and immersing in boiling water for six hours. After cooling, the mixture was extracted with ether and the dissolved material was incorporated in a measured amount of sesame oil as above. This fraction was labelled "bound" progesterone. In three instances the plasma after extraction with ether was tested for progesterone before hydrolysis.

The second method of separation was a modification of that used by Szego and Roberts (1947) for separating free and protein-bound estrogen. Here the plasma was added slowly with stirring to approximately ten volumes of acetone at 3°C. to precipitate the proteins. After standing overnight, the supernatant was pipetted off, and the precipitate was washed three times at the centrifuge with a 1:1 mixture of acetone and ether. An effort was made to keep the material cold. The washings were combined with the supernatant, a measured amount of sesame oil was added, and the ether and acetone were removed by distillation under diminished pressure. Most of the water of the plasma of course remained, and the oil was allowed to separate by standing or by centrifugation. This fraction was labelled "free" progesterone. The proteins were allowed to dry in air, powdered, and suspended or dissolved in distilled water. The suspension or solution was acidified with concentrated HCl to pH 0.5 to 1.0 and immersed in boiling water for six hours. After cooling, the mixture was extracted with ether, and the dissolved material was incorporated in sesame oil as described above. This fraction was labelled "bound" progesterone.

In certain instances raw plasma and raw whole blood were submitted to the same hydrolytic procedure without first removing the free progesterone. The objective was to determine whether the procedure destroys progesterone. In two instances the proteins in a sample of plasma were precipitated by tungstic acid, and the filtrate was assayed. In one instance a mass of plasma protein precipitated by acetone was suspended in water and tested without hydrolysis.

## RESULTS

The results are summarized in Table 1. The columns are arranged from left to right in the chronological order in which the samples were assayed; the increase in agreement reflects improvement in technical proficiency and greater foresight in making the dilutions for test. The failure to get exact values for the mouse blood was the result of not testing intermediate dilutions.

The values found for the concentration of progesterone in raw plasma ranged from 5.3 to 8.0 μg. per ml. The values for whole blood are less exact, but the apparent range was from 4.0 to 6.0 μg. per ml. The progesterone revealed by these tests appears to have been en-

tirely in the plasma. On the basis of the values for whole blood and the hematocrit for the second rabbit, the woman, and the monkey, plasma concentrations of 6.0, 5.4, and 6.5 $\mu$g. per ml., respectively, would be expected if all of the progesterone revealed were in the plasma. The

TABLE 1. BLOOD PROGESTERONE LEVELS, $\mu$G/CC.

| Species, status | Rabbit, pseudopregnant | | | | Mouse preg. ± | Man preg. 8 weeks | Monkey luteal phase |
|---|---|---|---|---|---|---|---|
| | 7th day | 7th day | 7th day | 6th day | | | |
| Hematocrit | 26 | 20 | 27 | | 27 | 35.7 | 28 |
| Raw whole blood | >2.6 <5.3 | 5.0 | | | 5–10 | 4.0 | 5.0 |
| Raw plasma | 5.3 | 6.0 | | 8.0 | 5–10 | 5.3 | 6.0 |
| Protein free filtrate, plasma | 3.7 | 5.0 | | | | | |
| Ether extract, plasma | 4.3 | 5.3 | | | 5–10 | 5.3 | 6.0 |
| Acetone supernatant, plasma | | | 6.6 | | 5–10 | 5.5 | 6.0 |
| Ether insol., plasma | 0 | 0 | | | | | 0 |
| Acetone ppt., plasma | | | 0 | | | | |
| Hydrolysate, ether insol. | 0.3 | 0.5 | | | 0.5 | 0.2 | 0.2 |
| Hydrolysate, acetone ppt. | | | 0.9 | | 0.8 | 0.2 | 0.2 |
| Hydrolysate, whole blood | | | | 6.0 | 7.3–9.3 | 4.3 | 5.3 |
| Hydrolysate, raw plasma | | 6.6–8.2 | | | 8.3–9.3 | 5.3 | 6.0 |
| Bound, per cent of total | 5.3 | 7.7 | 12.0 | | | 3.5 | 3.2 |

observed values of 6.0, 5.3, and 6.0 $\mu$g. per ml., respectively, are in satisfactory agreement with the expected values.

The values for the ethereal and acetone extracts are in good agreement with each other and with the values for the same raw plasmas. Apparently, therefore, the progesterone in plasma that manifested itself upon intrauterine injection was quantitatively extracted by both solvents. Moreover, neither solvent removed progesterone that was inactive while in the plasma.

Although tested only twice, and both times early in the study, the amount of active progesterone remaining in the filtrate of plasma after precipitation of the proteins was comparable to that in the raw plasma and that extracted by ether.

Tests of three plasma samples after removal of the ether-soluble material revealed no progesterone in concentrations as high as 0.33 $\mu$g. per ml. (the minimal concentration that the assay detects), and are in consequence recorded as being negative. Similarly, a suspension of acetone-precipitated plasma protein from the third rabbit exhibited no activity. When, however, the plasma after extraction with ether was submitted to acid hydrolysis and concentrated somewhat in oil solution, progesterone in amounts ranging from 0.2 to 0.5 $\mu$g. per ml. of original plasma was revealed. Similarly, acid hydrolysis of the plasma proteins precipitated by acetone revealed progesterone in amounts ranging from 0.2 to 0.9 $\mu$g. per ml. of original plasma.

If the progesterone extracted by ether and acetone is considered to be free progesterone and that revealing itself only after hydrolysis

of the proteins is considered to be bound, the latter fraction constituted 3.2 to 12.0 per cent of the total progesterone. The average was 6.5 per cent.

The low values for bound progesterone suggested the possibility that the procedure employed for hydrolysis of the proteins might inactivate bound progesterone almost as rapidly as it was freed. The quantity of progesterone found after hydrolysis of raw whole blood and raw plasma, however, was in every instance no less than the values for free progesterone in raw whole blood and in raw plasma. In some instances the values were almost exactly the sum of the free and bound fractions. Hence it seems unlikely that the hydrolytic procedures destroyed progesterone.

## DISCUSSION

The similarity of the levels of progesterone from species to species may represent nothing more than chance selection of physiological states with comparable levels of progesterone. The data suggest, however, that during active luteal function the blood levels of progesterone may be comparable in several species.

A plasma level of progesterone varying around 6.0 $\mu$g. per ml. is somewhat higher than the available estimates for other steroid hormones. McCullagh and Osborne (1938) found approximately 4 I.U. of androgenic activity in 100 ml. of human plasma. If the actuve substance be assumed to be testosterone, the concentration was 0.6 $\mu$g. per ml. In whole blood from bulls, Womack and Koch (1932) found 1 I.U. of androgenic activity in 600 ml., a concentration of 0.025 $\mu$g. per ml. if the active substance were testosterone. In blood from pregnant women the highest concentration of estrogen, calculated as estrone, found by Szego and Roberts (1947) was 0.9 $\mu$g. per ml., and by Goldberger and Frank (1942) was 0.13 $\mu$g. per ml. The higher level of progesterone is consistent with its lower activity if it is permissible to compare quite different actions of different hormones.

The finding that approximately 10 per cent of the total progesterone is "bound" contrasts with the observation that approximately 65 per cent of the estrogen in blood is protein-bound (Rakoff, Paschkis, and Cantarow, 1943; Szego and Roberts, 1946). Denaturation of protein by the procedures employed in removing the free progesterone is an obvious possibility to account for the low value for bound progesterone. Precipitation by cold acetone and the maintenance of low temperatures throughout the separation of the free fraction should minimize denaturation, however; the free values obtained by this method were similar to those given by extraction with ether and to the activity of untreated raw plasma. On the other hand, it is possible that all of the progesterone is bound to protein, most of it so loosely that the methods employed would not distinguish it from free progesterone. Such a circumstance would also be unlike that reported for estrogen in blood. It will be apparent that the objective

here was to compare the transport of progesterone with that reported for estrogen, and the observations cannot elucidate the nature of the association with protein. Indeed, the bound fraction may have been conjugated, for example as sulfate or glucuronide, and not in any way related to protein, inasmuch as the methods employed probably would not differentiate protein-bound and conjugated progesterone. A possible, but not insistent argument against conjugation is that conjugated progesterone has not apparently been reported to occur. An oblique argument that the binding was to protein is that this fraction was revealed by procedures used to demonstrate protein-bound estrogen, and estrogen has been separated from purified proteins (Roberts and Szego, 1946).

Protein-bound estrogen in blood has been suggested to act as a sort of reservoir, with an equilibrium between the free and protein-bound moieties (Szego and Roberts, 1946). This suggestion seems unlikely for bound progesterone. The amount of this fraction appears to be too low, relatively, and it seems incapable of freeing itself in a period of 48 hours in a uterine segment. Indeed, binding is apparently a means for the hepatic inactivation of progesterone in the mouse (Forbes and Hooker, unpublished). It would be desirable to know whether bound progesterone is dialyzable as is estrogen (Szego and Roberts, 1946). The low concentration of this fraction, the low solubility of progesterone in water below body temperature (Forbes and Hooker, 1948), and the rapid disappearance of progesterone from plasma stored at body temperature (Hooker and Forbes, unpublished) would make the determination difficult.

In the blood of the four species examined progesterone is thus apparently transported entirely in the plasma and is primarily free rather than bound to protein. The concentrations of free progesterone, and indeed of total progesterone, in no instance exceeded the solubility of this substance in water at body temperature (Forbes and Hooker, 1948). It is apparent, therefore, that no special or complex mechanism is needed to dissolve the amounts of progesterone found in blood, and none was indicated. Various mechanisms are possible and may be operating (Masson and Selye (1945) report a much higher solubility of progesterone in serum in vitro), but their necessity is not evident. The same situation appears to obtain for estrogen. The highest concentration found by Szego and Roberts (1947), calculated as estrone, was 0.9 μg. per ml. of whole blood, which could not be greater than 1.35 μg. per ml. of plasma (even assuming a hematocrit of 50), whereas estrone dissolves in water to the extent of 2.1 μg. per ml. (Doisy, 1939). The binding of roughly two-thirds of the total estrogen to protein (Rakoff, Paschkis, and Cantarow, 1943; Szego and Roberts, 1946) may serve to increase the solubility of this substance, but binding may be unnecessary for this purpose and, indeed, have quite another function.

Obviously, three values for progesterone in blood may be deter-

mined—free, bound, and total. The first may be learned by assaying raw blood or plasma, the last by assaying hydrolyzed raw blood or plasma, while determination of the bound fraction necessitates fractionation and hydrolysis. The labor and time involved are greatest when the bound value is determined and least when only the free value is wanted. If the desired end is an assessment of the humoral environment of a target organ, the free value appears at the moment to be the only value of importance. The significance of the bound fraction is unknown, but it has thus far been quite low, and of indicated consequence only in the metabolism of progesterone.

## SUMMARY

The blood levels of progesterone during one phase of luteal activity in rabbits, mice, a monkey, and a pregnant woman were determined by a bioassay method that detected a concentration of 0.33 $\mu$g. per ml. In the samples examined the total progesterone was between 4.0 and 8.0 $\mu$g. per ml. of whole blood, and was located entirely in the plasma.

Progesterone in the plasma apparently was present in two forms. Roughly 90 per cent was free, and the remainder was bound to protein or conjugated. The bound or conjugated fraction was inactive as tested, and was revealed only after partial hydrolysis of the plasma proteins. Fractionation was accomplished by methods that have been employed to separate free and protein-bound estrogen in blood.

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# THE EFFECT OF GROWTH HORMONE ON THE INORGANIC PHOSPHORUS LEVELS IN THE PLASMA[1]

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THE fact that the hypophysis may play an important role in the control of inorganic phosphorus in the animal has been known for some time. Kobayashi (1931) and Schijo (1934) have shown that hypophysectomy in dogs causes a reduction of the inorganic phosphorus of the blood. Irradiation of the rabbit's pituitary also brought about a decrease in blood phosphorus (Cannavo and Beninato, 1934). In rats, the removal of the pituitary reduces markedly the serum inorganic phosphorus level (Anderson and Castler, 1938; Jones and Shinowara, 1942).

The earlier work of Teel and Watkins (1929) indicated that anterior pituitary extract produced an increase of inorganic phosphorus in the blood of dogs. These results were later extended by Gerschman and Marenzi (1935) who found that the blood inorganic phosphorus level changes from 4.25 mg. to 7.88 mg. per cent as the result of pituitary extract injections. The clinical data of Reifenstein *et al.* (1946) showed that the serum inorganic phosphorus level is elevated in both acromegalic patients and growing children. These experiments seem to indicate that growth hormone in the anterior pituitary extract is responsible for the changes of inorganic phosphorus content in the plasma.

*Experimental.* Male rats of the Long-Evans strain were used. The animals, fasted from 16 to 20 hours, were anesthetized with sodium amytal and blood was taken from the inferior vena cava with heparin solution as the anticoagulative agent. The method of Berenblum and Chain (1938) was employed for phosphorus determination. Growth hormone was prepared by the method previously described (Li, Evans and Simpson, 1945).

*The change of plasma inorganic phosphorus level with age.* The in-

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organic phosphorus concentration in the plasma of male rats[3] at different ages is summarized in Table 1. It is evident that young growing rats have more inorganic phosphorus in their blood in comparison with that found in later stages of life. The inorganic phosphorus in the plasma is maintained at the same level from 15 days to 55 days of

TABLE 1. INORGANIC PHOSPHORUS CONTENT IN PLASMA OF MALE RATS AT DIFFERENT AGES

| Age | No. of rats | Body weight | Inorganic phosphorus |
|---|---|---|---|
| days | | gm. | mg. per 100 cc. plasma |
| 15 | 15 | 31.0 ± 0.92* | 10.6 ±0.08 |
| 27 | 9 | 59.2 ± 3.0 | 10.9 ±0.45 |
| 40 | 5 | 139.4 ± 0.8 | 10.7 ±0.55 |
| 55 | 5 | 217.6 ±18.5 | 11.4 ±0.57 |
| 68 | 6 | 222.3 ± 8.3 | 8.2 ±0.34 |
| 73 | 6 | 253.5 ± 9.4 | 8.2 ±0.42 |
| 82 | 6 | 264.7 ±17.9 | 7.8 ±0.28 |
| 119 | 5 | 330.0 ±18.5 | 7.7 ±0.31 |

* Mean ± standard deviation.

age. When the animals grow older, the level decreases from 11.4 to 7.7 mg. per 100 cc. plasma and then becomes constant.

*The effect of hypophysectomy.* In confirmation of earlier investigators, hypophysectomy causes a lowering of inorganic phosphorus content in the plasma of rats. It may be seen in Table 2 that the phosphorus concentration changes from 10.7 mg. to 8.1 mg. per 100 cc. plasma after the pituitary has been removed for 7 days. The inorganic phosphorus level continues to fall but becomes constant at 2 weeks postoperative.

*The influence of growth hormone.* Male rats were hypophysectomized at the age of 40 days; intraperitoneal injections of growth hor-

TABLE 2. INORGANIC PHOSPHORUS CONTENT IN PLASMA OF HYPOPHYSECTOMIZED MALE RATS AT DIFFERENT POSTOPERATIVE PERIODS
40 days old at operation

| Post-operative | No. of rats | Body weight | | Inorganic phosphorus |
|---|---|---|---|---|
| | | Initial | Final | |
| days | | gm. | gm. | mg. per 100 cc. plasma |
| 0 | 5 | 139.4 ±0.8* | | 10.7 ±0.55 |
| 7 | 4 | 140.0 ±6.8 | 118.2 ± 8.1 | 8.1 ±0.33 |
| 14 | 26 | 138.3 ±2.9 | 115.4 ± 3.4 | 6.6 ±0.22 |
| 28 | 7 | 141.2 ±3.0 | 118.6 ±10.0 | 5.6 ±0.20 |
| 42 | 3 | 145.0 ±6.2 | 115.7 ± 4.2 | 6.9 ±0.24 |

* Mean +standard deviation.

[3] The plasma inorganic phosphorus level of mature female rats was also determined and found somewhat lower than that occurring in male rats of similar age. From 17 female rats of ages from 91 to 111 days, an average value of 6.5 ±0.41 mg. inorganic phosphorus in 100 cc. plasma was obtained. As shown in Table 1, male rats 119 days old have 7.7 ±0.31 mg. per cent of plasma inorganic phosphorus.

TABLE 3. EFFECT OF GROWTH HORMONE ON INORGANIC PHOSPHORUS CONTENT IN PLASMA OF HYPOPHYSECTOMIZED MALE RATS*

| Daily dose | No. of rats | Body weight | | | Inorganic phosphorus |
|---|---|---|---|---|---|
| | | Initial | Final† | Gain | |
| mg. | | gm. | gm. | gm. | mg. per 100 cc. plasma |
| 0.00 | 26 | 138.3 ±2.9‡ | 115.4 ±3.4 | −22.9 | 6.6 ±0.22 |
| 0.05 | 5 | 144.6 ±5.3 | 152.0 ±1.5 | 7.4 | 8.2 ±0.41 |
| 0.10 | 8 | 145.5 ±4.1 | 150.4 ±4.6 | 3.9 | 10.6 ±0.37 |
| 0.20 | 8 | 134.8 ±2.6 | 146.0 ±2.6 | 11.2 | 10.3 ±0.36 |

* Rats were operated at 40 days of age; intraperitoneal injections began on the day of operation and lasted for 15 days.
† Final body weights were obtained just before fasting.
‡ Mean ± standard deviation.

mone began on the day of operation and were continued for 15 days. Table 3 shows that a daily dose of 0.10 mg. is sufficient to prevent the fall of the inorganic phosphorus concentration in the plasma and to keep it at a normal level. It is of note that doubling of this daily dose does not elevate the phosphorus level above the initial level. However, 0.05 mg. of the hormone injected daily for 15 days will give a higher plasma inorganic phosphorus content than that in the controls.

TABLE 4. EFFECT OF CHRONIC INJECTION OF GROWTH HORMONE ON INORGANIC PHOSPHORUS CONTENT IN PLASMA OF HYPOPHYSECTOMIZED FEMALE RATS

| Experiment | Body weight | | | Inorganic phosphorus |
|---|---|---|---|---|
| | Initial | Final | Gain | |
| | gm. | gm. | gm. | mg. per 100 cc. plasma |
| Injected* | 68.6 ±0.54† | 409.6 ±27.6 | 341.0 | 5.2 ±0.41 |
| Control | 66.0 ±0.50 | 103.6 ± 6.3 | 37.6 | 2.7 ±0.20 |

* Rats 26 to 28 days of age at operation; 12 days postoperative at the beginning of injection; 0.1 mg. daily dose for the first 111 days, 0.2 mg. 270 days, and 0.4 mg. the last 22 days.
† Mean ± standard deviation.

In another experiment, hypophysectomized female rats were injected with growth hormone for 437 days; the plasma of these animals was analyzed for inorganic phosphorus. Results are summarized in Table 4. The control group gained 37.5 gm. having an inorganic phosphorus content of 2.7 mg. per 100 cc. plasma, while the injected animals gained 341.0 gm. and the plasma inorganic phosphorus level almost doubled that of the control.

Since young growing animals have a higher inorganic phosphorus concentration in the plasma and since hypophysectomy causes a stoppage of body growth with an accompanied lowering of the phosphorus level, the plasma inorganic phosphorus content may therefore be

taken as an indication of growth. The augmenting effect of growth hormone is hence to be anticipated. Until the actual significance of inorganic phosphorus on the process of growth is known, we offer no interpretation for the results herein reported.

## SUMMARY

The inorganic phosphorus level in the rat plasma is found to decrease with age. Hypophysectomy causes a decrease of the plasma inorganic phosphorus content, while injections with growth hormone prevent this fall and even elevate the phosphorus level above that of the control.

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# INFLUENCE OF GROWTH AND ADRENOCORTICOTROPIC HORMONES ON THE BODY COMPOSITION OF HYPOPHYSECTOMIZED RATS[1]

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## A. THE INFLUENCE OF GROWTH HORMONE

THE changes in the body composition of animals after treatment with pituitary growth extracts have been studied by several investigators. Both Downs (1930) and Wadehn (1932) obtained accelerated growth in mice with a pituitary preparation and found that the treated animals contained more water and protein but less fat than did the controls. Bierring and Nielsen (1932) induced increase in body weight in rats by an alkaline anterior pituitary extract and showed that the body weight increment was not entirely due to retention of water. Using the pair-fed technique, Lee and Schaffer (1934) conclusively demonstrated that the growth in rats caused by injection of pituitary growth extract is chiefly due to increase in the protein and water content and that there is a decrease in fat. Recent studies of Young (1945) have confirmed these findings.

Since all preceding experiments have been carried out with only partially purified growth-promoting extracts, it is naturally difficult to decide as to whether such results were due to the growth hormone itself or to other contaminating proteins. In addition, earlier investigators did not employ hypophysectomized animals. The results obtained might conceivably have been caused by the influence of the injected extract upon the animals' pituitary. For these reasons, we have determined again the chief chemical constituents—water, lipids and protein of the carcass of hypophysectomized rats treated with the pure anterior hypophyseal growth hormone as isolated by Li *et al.* (1945).

### EXPERIMENTAL

Female rats, 26 days old at hypophysectomy and 26 days postoperative, were restricted to a daily intake of approximately 3.0 gms. of food and injected with 0.10 mg. of the growth hormone daily in-

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traperitoneally for 30 days. Before the animals were autopsied, they were fasted for 24 hours. The entire gastrointestinal tract and internal organs were removed and discarded. The carcass was weighed and frozen in a cold room kept at −20°C. Moisture was determined by drying the frozen carcass in vacuum to constant weight. The dried carcass was then ground and the resulting uniform mixture was used for the determination of the total lipids and of total nitrogen. The fat was extracted thoroughly by means of petroleum ether in a Soxhlet apparatus. The ether extract was evaporated and the residue dried to constant weight. The weight of the dry residue was taken as the measure of the total fat in the tissue. The nitrogen content of the carcass was determined by the micro-Kjeldahl method, a factor of 6.25 being used for conversion into the protein value.

## RESULTS

As shown in Table 1, the average gain in body weight of 7 treated animals was 42.5 gms., whereas that of the 7 controls was only 8.9 gms. The composition of the carcass is altered by the growth hormone treatment. Data in Table 1 indicates that the carcass of the treated

TABLE 1. COMPOSITION OF THE CARCASS OF HYPOPHYSECTOMIZED FEMALE RATS UNDER RESTRICTED FOOD INTAKE AFTER GROWTH HORMONE TREATMENT
7 animals in each experiment

| Experiment | Body weight | | Weight per 100 gm. carcass | | |
|---|---|---|---|---|---|
| | Initial | Autopsy | Water | Fat | Protein |
| Injected[1] | 61.6 ±1.9[2] | 104.1 ±1.9 | 70.32 ±0.68 (<0.001)[3] | 4.44 ±0.41 (<0.01) | 19.24 ±0.39 (<0.02) |
| Control | 63.7 ±2.2 | 72.6 ±1.6 | 66.58 ±0.84 | 9.39 ±0.96 | 17.80 ±0.14 |

[1] Hypophysectomized female rats, 26 to 28 days old at operation and 26 days post-operative, were restricted to approximately 3 gms. food intake daily. A daily dose of 0.1 mg. growth hormone was used for 30 days.
[2] Mean ± standard deviation.
[3] Fisher's p values.

animals contained more water and protein but less fat as compared with controls. For instance, the total lipids were reduced from 9.39 gms. to 4.44 gms. per 100 gms. tissue, while the protein content increased from 17.80 gms. to 19.24 gms. The retention of water is also marked; it changed from 66.58 to 70.32 per cent. These differences in the percentage composition of the tissues between injected and control rats were all highly significant as analyzed by the statistical method of Fisher (1938).

In another experiment, male rats hypophysectomized at 40 days of age were used. Intraperitoneal daily injections of 0.2 mg. growth hormone began on the day of operation and continued for 15 days. The animals were allowed to eat ad libitum but were fasted 24 hours before autopsy. It may be seen in Table 2 that the uninjected (control) animals lost 13.4 gms. in body weight while the animals receiving growth hormone gained 22.0 gms. The fat content in the carcass of

the treated animals was significantly lower than that in the controls: the value being 5.70 gms. versus 8.66 gms. per 100 gms. tissue. The percentage content of protein is somewhat higher in the treated carcass but not statistically significant. Furthermore, the amount of

TABLE 2. COMPOSITION OF THE CARCASS OF HYPOPHYSECTOMIZED MALE RATS AFTER INJECTIONS OF GROWTH HORMONE

| Experiment | No. of rats | Body weight | | Weight per 100 gm. carcass | | |
|---|---|---|---|---|---|---|
| | | Onset | Autopsy | Water | Fat | Protein |
| | | gm. | gm. | | | |
| Growth Hormone[1] | 8 | 126.0±1.0[2] | 148.0±0.3 | 70.55±0.30 (<0.001)[3] | 5.70±0.38 (<0.01) | 18.70±0.27 (0.20) |
| Control | 12 | 125.4±1.5 | 112.0±2.9 | 66.20±0.41 | 8.66±0.66 | 17.72±0.54 |

[1] [illegible] at 40 days of age, were injected with 0.20 mg. growth hormone daily for 15 [illegible] tion; animals were fasted 24 hours before autopsy.
[2] [illegible]
[3] [illegible]

water in the treated rats is highly increased. Thus, the results with male animals are essentially the same as those obtained with hypophysectomized female rats.

## DISCUSSION

One of the characteristic functions of growth hormone is to retain nitrogen either in normal rats, in diabetic rats, or in rats with bilateral femoral fractures (Li, and Evans, 1947). It has further been shown that growth hormone causes a decrease of free amino acid in the plasma of rats (Li, unpublished data). It seems therefore reasonable to assume that the body growth induced by growth hormone treatment is at least partly due to the result of the acceleration of protein synthesis. The results just presented are in line with this assumption. The data show that *growth hormone causes a rise in the protein and water content with a lowering of the fat content of the body and* it is immaterial whether the animals are restricted in food intake or allowed to eat ad libitum. In fact, Young (1945) has shown that a restriction in food intake exaggerates the changes in body composition which normally follow pituitary treatment in the rat.

Since the rapid accumulation of protein is a distinctive pattern occurring in the process of growth (Moulton, 1923), there is no doubt that *the gain in body weight induced by growth hormone injections represents true growth*. It is well known that embryonic tissues containing high proportion of water and are relatively low in fat; it may be inferred that the tissue formed by the action of growth hormone is embryonic in nature.

Although it is clear that the total fat content of the body is reduced as the result of growth hormone injections, the nature of these changes remain unknown. The lowering of the fat content by growth hormone may reflect only a reduction in neutral fat, for it has been shown (Williams, Galbraith, Kaucher and Macy, 1945) that about 90 per cent of the total fat in rats consists of triglycerides. Since structural lipids, such as phospholipid, cholesterol and cerebrocides, are

essential components of cellular structure and since some of these lipids are known to increase with growth (Williams, Galbraith, Kaucher and Macy, 1945; Williams, Galbraith, Kaucher, Moyer Richards and Macy 1945), it may be anticipated that further studies will show that the concentration of "structural" lipids in tissues may actually be increased by growth hormone injections.

### B. THE INFLUENCE OF ADRENOCORTICOTROPIC HORMONE

Evidence has accumulated indicating that adrenocorticotropic hormone may be regarded as a specific growth-inhibiting substance (Li and Evans, 1947). When it was injected simultaneously with growth hormone in hypophysectomized rats a counteraction exists between these two hormones (Marx, Simpson, Li and Evans, 1943). Since growth hormone has been shown to cause an increase in the protein content and a reduction in the fat in the body of hypophysectomized rats, it was of interest to similarly investigate the body composition of animals after adrenocorticotropic hormone treatment.

### EXPERIMENTAL

Female rats, hypophysectomized on 26 days of age and 26 days postoperative, were injected intraperitoneally twice daily with a 1.0 mg. total daily dose of pure adrenocorticotropic hormone for 10 days. The animals were restricted to approximately 4.5 gm. of diet daily and fasted 24 hours before autopsy. The hormone was prepared by the method previously described (Li, Simpson and Evans, 1943). The carcass analysis was carried out in the same manner as outlined in the growth hormone experiment.

### RESULTS

The body weight and its chemical composition of experimental and control rats are summarized in Table 3. As to be expected, there is no essential difference in body weight between the experimental and control groups. The changes in body composition are due to the loss of water and an increase of fat content from 8.62 to 11.57 gms. per 100 gms. carcass. The protein content is practically unchanged. It may be noted that the changes in fat and water content are statistically significant.

TABLE 3. COMPOSITION OF THE CARCASS OF HYPOPHYSECTOMIZED FEMALE RATS AFTER INJECTIONS OF ADRENOCORTICOTROPIC HORMONE (ACTH)

| Experiment | No of rats | Body weight | | Weight per 100 gm. carcass | | |
|---|---|---|---|---|---|---|
| | | Onset | Autopsy | Water | Fat | Protein |
| | | gm. | gm. | | | |
| ACTH[1] | 6 | $74.3 \pm 2.1$[2] | $71.3 \pm 1.7$ | $64.55 \pm 0.52$ | $11.57 \pm 0.61$ | $16.89 \pm 0.32$ |
| | | | | $(<0.01)$[3] | $(<0.01)$ | |
| Control | 8 | $79.6 \pm 2.1$ | $76.5 \pm 1.5$ | $67.96 \pm 0.44$ | $8.62 \pm 0.39$ | $16.73 \pm 0.43$ |

[1] Female rats, hypophysectomized on 26 days of age and 26 days postoperative, were injected twice daily with 1.0 mg. total daily dose for 10 days; animals were under restricted food intake (approximately 4.5 gm. daily) and fasted 24 hours before autopsy.
[2] Mean ± standard deviation.
[3] Fisher's p values.

### DISCUSSION

Since the secretory activity of the adrenal cortex enhances protein catabolism (Li and Evans, 1947; Ingle, 1944). it may not be unreasonable to expect that the body of adrenocorticotropic hormone treated animals would contain less protein. Experiments just reported show no change in protein content of the treated rats, although the animals definitely deposit more fat. It may be that the carcass contains actually less protein but our determinations did not differentiate protein from non-protein nitrogen.

The analytical values recorded in Table 3 clearly demonstrate that *adrenocorticotropic hormone induces a rise in the fat content of the carcass with a diminution in the water content.* These changes are in contrast with those obtained with growth hormone. In view of the fact that adrenocorticotropic hormone is known to be a growth inhibitor, these opposite alterations in the body composition after treatment with these two hormones are not surprising.

### C. SUMMARY

Hypophysectomized rats, fed a limited quantity of food, were treated with either growth or adrenocorticotropic hormone. Analysis of the whole carcass showed that the growth hormone caused an increase in the protein and water content with a lowering of the fat content. On the other hand, the adrenocorticotropic hormone treatment resulted in a decrease in the water content and a gain in the fat content of the whole body.

### ACKNOWLEDGMENT

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# RENAL EXCRETION OF WATER AND ANTI-DIURETIC SUBSTANCES IN PATIENTS WITH HEPATIC CIRRHOSIS AND RATS WITH DIETARY LIVER INJURY[1]

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THE problem of water retention in liver disease has been approached through both clinical and animal studies. In clinical studies Aldersberg and Fox (1943) found an abnormal retention of water when water tolerance tests were performed on patients with severe parenchymatous liver disease and recently Ralli *et al.* (1945) have demonstrated antidiuretic activity in urines from patients with hepatic cirrhosis and ascites. With respect to animal studies Aldersberg and Fox (1943) observed abnormal water tolerance tests in dogs when the liver was injured by phosphorus and histamine. Shay, Kolm, and Fels (1945) also reported the occurrence of abnormal water tolerance tests in rats fed a high fat diet. Leslie and Ralli (1947) have recently found that rats fed a high fat-low protein diet showed a decreased urine output during water tolerance tests, and they observed that the urines from such rats had antidiuretic activity.

The present study is to report some observations on the antidiuretic activity of urine from patients with cirrhosis of the liver and ascites and to observe the effect of dietary liver injury in rats on water tolerance tests and the excretion of urinary antidiuretic material.

## METHODS

Seven 24 hour collections of urine were obtained from four patients with hepatic cirrhosis and ascites, and from four normal subjects. Each 24 hour urine collection was prepared for antidiuretic assay by the method of Ralli *et al.* (1945).

The liver injury was produced in rats by dietary means, as follows: Male, Sprague-Dawley rats weighing 130–170 grams, were placed on a diet of 16% casein, 51% lard, 30% corn starch, 3% salt mixture, and adequate amounts

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of vitamins A, D and the vitamin B complex for 200 days, as previously described (Hall and Drill, 1948). Groups of 10 animals each received the above diet alone or plus supplements of either casein, yeast, liver extract, ventriculin, methionine, or choline. This diet without the special supplements resulted in a marked fatty change and fibrosis in the liver which could be prevented by choline or liver extract (Hall and Drill, 1948).

While on the high-fat diet (51% fat—16% casein) a 24 hour urine specimen was collected from the 10 animals of each group on the 65th and 160th day of the study. During the collection period water was allowed *ad libitum*, but no food was given. The urine from each group of 10 rats was prepared for assay of antidiuretic substance (hereafter referred to as ADS) by the same method used for the patients' urines (Ralli *et al.* 1945). The processed urine from each group of 10 rats was adjusted to a final volume of 20 cc. for assay. Water tolerance tests were made on all of the groups of animals after the 51% fat diet had been fed for 185 to 200 days. Each group was divided into subgroups of three animals each, and two water tolerance tests were made on each sub-group.

The rats with hepatic injury, induced by the 51% fat diet, were compared with normal control animals. The normal control rats received a synthetic diet similar in all respects to the high fat diet, except that the amount of fat was reduced to 6% and the corn starch increased to 75%, the casein content remaining at 16%. These control animals received the same treatment in the collection of urine for ADS assay and for water tolerance tests as described above the for animals fed the high (51%) fat diet. Such urine collections and tolerance tests were also made at the same time as those performed on the rats receiving the high fat diet. Two 24 hour collections of urine were also made from a group of 10 rats fed a laboratory chow diet for further comparison of the ADS assay.

Both the human and rat urines were assayed for antidiuretic activity by the method of Ham and Landis (1942). The urine assays were performed on stock male rats, weighing between 200 and 300 grams, and fed a laboratory chow diet. Food was removed from the cages of the assay rats four to five hours before the test, but water was permitted up to the time of the initial hydration. The animals then received an initial hydration, 2.5% of body weight, with 0.2% NaCl. Two hours later a second hydrating dose of NaCl, equivalent to 5% of their body weight was administered. The urine extracts to be tested were injected intraperitoneally, 1 cc. per 100 grams of body weight, at the time of the second hydration. A group of control rats, similarly hydrated, received the same dose of distilled water intraperitoneally. A second group of control hydrated rats received 5 milliunits of pituitrin per cc. per 100 grams of body weight intraperitoneally. Each assay was performed on groups of three animals, and urine measurements, made every 15 minutes for 3 hours, were timed from the second hydration.

The water tolerance tests were also performed on groups of three animals, using two hydrating doses of 0.2% NaCl, as described above. Urine excretions were then measured every 15 minutes for three hours following the second hydration. In the water tolerance tests normal animals fed the 16% casein—5% fat diet and others fed the laboratory chow diet served as controls for rats receiving the high-fat diet (16% casein—51% fat).

The degree of liver injury noted in the tables was determined during the

experiment by histological study of biopsy specimens, and after 200 days by both histological study and total fat analyses.

## RESULTS

The urines from the patients with cirrhosis showed an antidiuretic effect while those from normal subjects did not, as illustrated in a typical assay in Figure 1. The area under each graph of urine output may be measured in square inches. The difference in urine output between the water injected controls and animals injected with urine extracts or

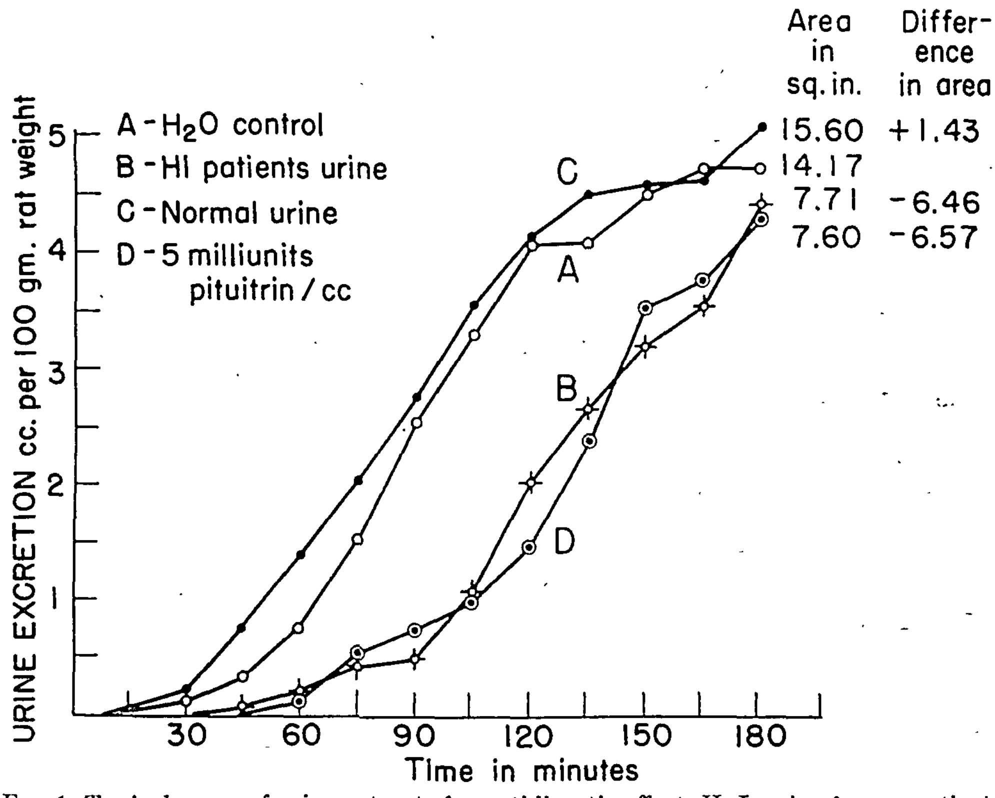


FIG. 1. Typical assay of urine extracts for antidiuretic effect. H. I. urine from a patient with hepatic cirrhosis and ascites.

pituitrin may then be recorded as a difference in square inches, a negative value indicating a lesser output of urine, and thus an antidiuretic effect of the injected material. Such assays on the human urines are summarized in Table 1. The ADS from the urine of patients with cirrhosis has an antidiuretic activity approximating that of 5 milliunits of pituitrin.

The urines from the rats fed the high-fat diet had antidiuretic activity, but an effect of the same magnitude was also obtained from the urines from animals fed the normal fat diet. Further, the antidiuretic effect was not related to the absence, presence or degree of liver injury in the different groups. The two urine samples collected from the animals fed the laboratory chow diet also showed antidiuretic activity (Table 2). The antidiuretic effect of urines from animals on

TABLE 1. ANTIDIURETIC ACTIVITY OF HUMAN URINE

| Urine sample | Test No. | $H_2O$ control in sq. inches | Pituitrin 5 mu | Urine assay |
|---|---|---|---|---|
| Normal subjects | | | | |
| I | | 11.82 | −2.90 | +0.48 |
| II | | 16.43 | | −1.68 |
| III | | 13.34 | −7.98 | −1.26 |
| IV | | 14.17 | −6.57 | +1.43 |
| Patients with cirrhosis and ascites | | | | |
| I* | 1 | 11.82 | −2.90 | −4.54 |
| | 2 | 12.20 | −5.65 | −2.91 |
| | 3 | 17.45 | −7.90 | −7.90 |
| II* | 1 | 17.45 | −7.90 | −5.14 |
| | 2 | 12.60 | −7.26 | −5.06 |
| | 3 | 13.94 | −5.59 | −3.10 |
| III* | 1 | 17.40 | −6.45 | −5.80 |
| | 2 | 17.40 | −6.45 | −6.86 |
| | 3 | 19.86 | −11.60 | −13.96 |
| IV* | 1 | 14.17 | −6.57 | −7.72 |
| V | 1 | 17.40 | −6.45 | −4.95 |
| VI | 1 | 17.80 | | −3.79 |
| VII | 1 | 17.25 | | −4.20 |

* Samples from the same patient.

TABLE 2. ANTIDIURETIC ACTIVITY OF RAT URINE

| Test No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Urine sample | 65th day | 65th day | 160th day | 160th day | | |
| $H_2O$ Control | 7.37 | 10.58 | 14.95 | 17.09 | 11.57 | 18.35 |
| Groups with no hepatic disease | | | | | | |
| N.F. Diet | | −7.34 | | −14.92 | | |
| H.F.+Liver Ext. | | −8.33 | | − 8.73 | | |
| H.F.+Choline | | −4.87 | −12.48 | | | |
| Groups with markedly fatty livers and fibrosis | | | | | | |
| H.F. alone | +0.84 | | | −6.45 | | |
| H.F.+Ventriculin | −1.44 | | −11.91 | | | |
| Groups with intermediate stages of fatty livers | | | | | | |
| H.F.+Casein | −0.62 | | − 9.91 | | | |
| H.F.+Yeast | +1.41 | | | −12.49 | | |
| H.F.+Methionine | | −3.69 | −10.10 | | | |
| Animals on laboratory chow diet | | | | | | |
| | | | | | −5.17 | −10.68 |

N.F. = normal fat diet (6% fat—16% casein).
H.F. = high fat diet (51% fat—16% casein).
$H_2O$ control results expressed in square inches. Other groups are recorded as difference in sq. in. from water control group.

both normal and high-fat diets was not significantly altered when the urines were concentrated further or if they were diluted to their original volume of collection, or if the sediment was removed. Dialysis of the urines for 24 hrs did not remove the antidiuretic substance.

The tolerance tests also failed to show any difference in water retention between those animals on a high-fat diet, even though marked hepatic changes were produced, and animals fed a normal-fat diet (Table 3). The results in Table 3 are expressed in square inches instead of differences in square inches because the groups on the synthetic diet were more comparable to one another than to the animals fed the laboratory chow diet. Figure 2 illustrates the urine output in a typical assay. It can be seen in Figure 2 that no difference exists between the urine output of the groups even if the endpoint is taken as the time to excrete 50% of the hydrating volume of fluid. This was true of all of the tolerance tests.

TABLE 3. WATER TOLERANCE TESTS ON RATS AFTER 185–200 DAYS OF A HIGH FAT DIET. RESULTS IN SQUARE INCHES

| Test No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Mean |
|---|---|---|---|---|---|---|---|---|
| Control, Chow Fed | 17.17 | 13.83 | 10.95 | 12.33 | 18.58 | 19.42 | 15.57 | 15.41 |
| Groups with no liver disease | | | | | | | | |
| N.F. | | | | 16.21 | | 13.85 | | 15.03 |
| H.F. + Liver Ext. | 15.35 | 16.80 | 15.28 | 15.83 | 14.75 | | 17.04 | 15.84 |
| H.F. + Choline | 12.79 | | 13.25 | 14.70 | | 11.99 | | 13.18 |
| Groups with markedly fatty livers and fibrosis | | | | | | | | |
| H.F. alone | | 16.66 | | 15.22 | 18.68 | 14.33 | | 16.22 |
| H.F. + Ventriculin | 15.04 | 12.00 | 14.41 | 14.95 | 16.41 | | 12.62 | 14.24 |
| Groups with intermediate stages of fatty livers | | | | | | | | |
| H.F. + Casein | 12.84 | 14.22 | 14.54 | | 12.43 | 14.20 | 15.51 | 13.96 |
| H.F. + Yeast | 15.90 | 13.71 | 15.49 | | 13.72 | 16.16 | 15.94 | 15.15 |
| H.F. + Methionine | 14.53 | 14.79 | 17.10 | | 12.84 | 14.03 | 17.67 | 15.16 |

## DISCUSSION

The urines from the patients with cirrhosis and ascites had an antidiuretic effect which was comparable to that produced by 5 milliunits of pituitrin. This confirms the findings of Ralli *et al.* (1945) that in the presence of ascites the urines from patients with cirrhosis contain an antidiuretic substance. This antidiuretic effect was absent in the four normal urines that were tested. No attempt was made here to observe any correlation between the severity of the cirrhosis or the presence or absence of ascites and the antidiuretic potency of the urine. The patients used had advanced cirrhosis and ascites.

However, the rats with hepatic disease failed to show any abnormality of water metabolism in response to water tolerance tests or

assay for urinary ADS. The urine samples from rats with fibrotic and markedly fatty livers were antidiuretic, but no more so than urines from rats on a high fat diet in whom the liver injury was prevented by choline or liver extract, or in rats fed a low-fat diet or a labora-

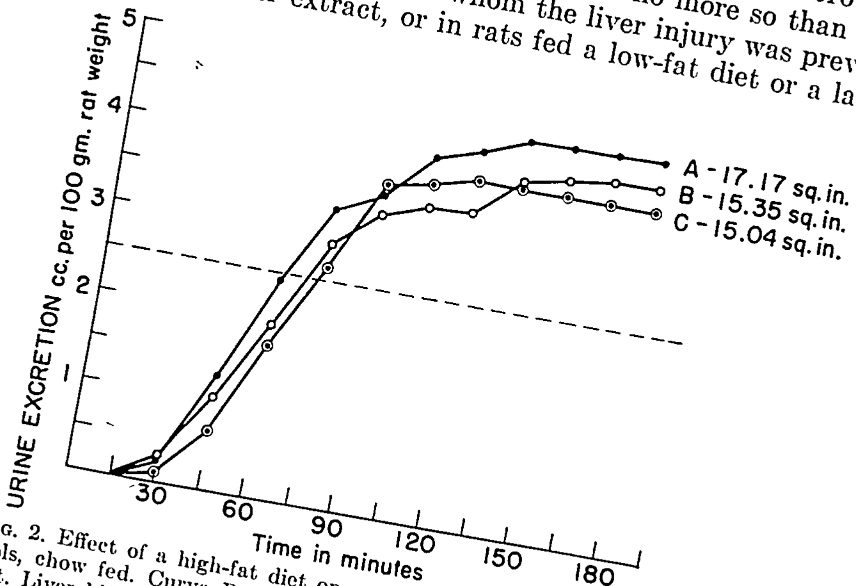


FIG. 2. Effect of a high-fat diet on water tolerance tests in rats. Curve A, water controls, chow fed. Curve B, animals fed a high-fat diet plus supplements of liver extract. Liver histology normal. Curve C, animals fed a high-fat diet. Liver showed fatty change and fibrosis. Broken horizontal indicates 50% excretion.

tory chow diet. Other investigators have found the urines from normal, well hydrated rats to be free of antidiuretic activity when assayed by methods similar to those used in the present study (Leslie and Ralli, 1947; Gilman and Goodman, 1937). The basis for this discrepancy is not clear at the present time. One other group found urines from rats fed a normal stock diet as well as those fed a high-fat diet to be free of urinary ADS (Birnie, Eversole and Gaunt, 1948).

The finding of normal tolerance tests in spite of severe hepatic injury supports the conclusions derived from the ADS determinations. Neither the high fat diet nor the fatty change and fibrosis in the liver altered the diuresis. These findings are not at variance with those of others. Shay, Kolm and Fels (1945) found that water tolerance tests were normal on animals fed a high-fat diet if they were allowed water up to the time of testing, *but were abnormal if water was withheld for 16 hours*. Dehydration alone in normal rats will produce an antidiuretic substance in rat urine (Gilman and Goodman, 1937). The animals in the present study were not dehydrated. Leslie and Ralli (1947) also withheld water (overnight) prior to the tolerance tests. They obtained the greatest retention if the diet was free of added NaCl as well as being high in fat and low in protein. Both groups of workers found abnormal tests as early as 7 days. This is very early for any marked

degree of hepatic injury by the diets used, although functional changes may have taken place. However, we found normal water tolerance tests in animals that had severe hepatic disease, who, however, were not dehydrated prior to the tolerance test. Findings similar to ours were obtained by Birnie, Eversole and Gaunt (1948). They noted that a high-fat diet did not influence the water diuresis during hydration tests. If their animals were dehydrated before the test, they obtained some indications in agreement with other workers, that fat-fed animals excreted water a little less readily than controls, but the effect was not entirely consistent.

It appears then that the fluid retention reported in rats may depend more on the state of hydration than on the presence of liver disease alone, but may be a combination of the effects of dehydration in the presence of liver disease or an abnormal diet. It is also possible that the diets used by Shay, Kolm and Fels (1945) and by Leslie and Ralli (1947) may produce somewhat different liver lesions than in the present study or may in some way make the animals more sensitive to the effects of dehydration. The increased sensitivity of rats with liver injury to the effects of dehydration seems to be the more likely possibility. As Leslie and Ralli (1947) have pointed out, such rats may also show an abnormal renal function as indicated by PSP tests, and in the presence of liver injury and/or dehydration, the renal change may be important in such studies of water tolerance and urinary antidiuretic substance.

## SUMMARY

Urine extracts from patients with hepatic cirrhosis and ascites had antidiuretic activity when assayed on rats, whereas similar urine extracts from normal subjects were devoid of such activity.

Urine extracts prepared from rats with liver damage or without liver damage all showed antidiuretic activity. No difference in the intensity of the antidiuretic effect was noted in the absence or presence of liver injury.

Normal water tolerance tests were obtained in rats that had fatty and fibrotic livers. The possible relationship of any pretreatment, such as dehydration, to the tolerance test in the presence of liver injury is discussed.

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# ANDROGEN: KETOSTEROID RATIOS OF RABBIT URINE[1]

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SIMULTANEOUS measurements of biologically active androgens and of 17-ketosteroids have been reported for humans and monkeys. Active androgens account for 30% to 60% of the netural ketonic fraction of human urine (Bauman and Metzger, 1940) and for about 9% to 13% of the same fraction of monkeys' urine, in terms of androsterone (Dorfman *et al.*, 1947). These figures do not represent the actual proportions in untreated urine since considerable alteration of the original steroids occurs during acid hydrolysis (Venning *et al.*, 1942).

Urinary 17-ketosteroid levels have been determined for male rabbits (Kimeldorf, 1948) and for female rabbits (De Koning *et al.*, 1948). Metabolites obtained from in vitro incubation of testosterone with rabbit liver slices have been investigated by Clark and Kochakian (1947) who could isolate neither androsterone nor its isomers from the products.

Studies on other animals show that biologically active androgens are excreted in relatively small amounts by the chimpanzee (Fish *et al.*, 1941), by the rat and dog (Clark and Kochakian, 1947), and by the bull (Hooker, 1937).

In the present study, the biologically active androgens of male and female rabbit urine extracts assayed by the chick comb method have been compared to colorimetric values of 17-ketosteroids in the same extracts.

## METHODS

Three normal male and three normal female rabbits of the New Zealand strain were used. Forty-eight hour urine samples were collected over 5 cc. of concentrated hydrochloric acid, refluxed for seven minutes with concentrated hydrochloric acid (15% by volume), extracted with ether. Then the ether was washed several times with saturated sodium carbonate, with two normal sodium hydroxide, and with distilled water, to obtain the crude neutral fraction. This extract either was assayed directly or was treated with Girard's reagent T yielding the neutral ketonic fraction (Pincus, 1945).

Colorimetric analyses were run on the Beckman DU Spectophotometer

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[2] The authors wish to acknowledge the assistance of J. De Koning who prepared some of the urine extracts.

TABLE 3. RECOVERY OF ANDROSTERONE IN RABBIT URINE EXTRACTS

| Androsterone mg. | Added to | 17-ketosteroids in unspiked aliquot mg. | 17-ketosteroids in spiked aliquot mg. | Androgens in unspiked aliquot mg. | Androgens in spiked aliquot mg. | Recovery of 17-ketosteroids % | Recovery of androsterone % |
|---|---|---|---|---|---|---|---|
| 3.6 | Neutral ketonic fraction | 5.0 | 8.6 | 0.03 | 3.69 | — | 103 |
| 1.4 | Neutral ketonic fraction | 4.1 | 5.5 | 0.02 | 1.32 | — | 93 |
| 1.4 | Neutral ketonic fraction | 3.5 | 4.9 | 0.01 | 1.42 | — | 99 |
| 1.4 | Neutral ketonic fraction | 4.1 | 5.5 | 0.02 | 1.52 | — | 116 |
| 3.1 | Urine before hydrolysis | 2.8 | 6.0 | 0.04 | 1.35 | 102 | 42 |
| 3.1 | Urine before hydrolysis | 2.6 | 5.5 | 0.00 | 1.07 | 94 | 34 |
| 3.1 | Urine before hydrolysis | 3.2 | 5.7 | 0.12 | 1.05 | 82 | 30 |

Since the beta-ketosteroid fraction was not present in detectable amounts (Table 4), neither dehydroisoandrosterone nor isoandrosterone apparently are excreted in appreciable quantities by normal rabbits. (Similarly, no digitonin-precipitable ketosteroids were found in normal guinea pig urine by Miller and Dorfman, 1945.)

The proportions of alcoholic and non-alcoholic ketosteroids (Table 4) do not represent those originally present in the urine since acid hydrolysis causes a large conversion of alcoholic ketosteroids to non-alcoholic ketosteroids (Pincus and Pearlman, 1941).

TABLE 4. ALPHA & BETA, OH & NON-OH KETOSTEROIDS IN MALE RABBIT URINE EXTRACTS

| Rabbit's age months | Urine volume cc. | Alpha-ketosteroids mg. | Beta-ketosteroids mg. | Alcoholic ketosteroids mg. | Non-alcoholic ketosteroids mg. |
|---|---|---|---|---|---|
| 7 | 470 | 2.9 | 0 | — | — |
| | 535 | 2.9 | 0 | — | — |
| | 525 | — | — | 0.89 | 0.63 |
| 12 | 255 | 3.2 | 0 | — | — |
| | 300 | 3.6 | 0 | — | — |
| | 225 | — | — | 0.68 | 0.75 |
| 16 | 470 | 2.9 | 0 | — | — |
| | 500 | 2.1 | 0 | — | — |
| | 400 | — | — | 0.78 | 0.66 |

The exact chemical nature of the neutral 17-ketosteroids excreted by rabbits must await their separation and identification.

## SUMMARY

Low androgenic titers are found in rabbit urine extracts. In terms of androsterone, only about 2% of the neutral ketonic fraction (less than 1% of female) rabbit urine is biologically active. Recovery experiments and comparative data indicate that the proportion of androgens in untreated rabbits' urine, while probably considerably

TABLE 1. ANDROGENS AND 17-KETOSTEROIDS IN MALE RABBIT URINE EXTRACTS

| Rabbit's age months | Urine volume cc. | Fraction | Androgens as androsterone mg. | 17-ketosteroids mg. | % androgens in extract |
|---|---|---|---|---|---|
| 7 | 425 | Neutral ketonic | 0.02 | 2.4 | 1 |
| | 450 | Neutral ketonic | 0.03 | 4.3 | 1 |
| | 155 | Crude neutral | 0.00 | 1.4 | 0 |
| | 480 | Crude neutral | 0.01 | 3.6 | 0 |
| 12 | 160 | Neutral ketonic | 0.09 | 2.8 | 3 |
| | 240 | Neutral ketonic | 0.12 | 5.8 | 2 |
| | 95 | Crude neutral | 0.04 | 1.5 | 3 |
| | 225 | Crude neutral | 0.01 | 4.2 | 0 |
| 16 | 205 | Neutral ketonic | 0.12 | 2.4 | 5 |
| | 385 | Neutral ketonic | 0.05 | 2.8 | 2 |
| | 290 | Crude neutral | 0.01 | 3.2 | 0 |
| (Average male) | 310 | Neutral ketonic | 0.07 | 3.4 | 2 |

proportion of any androsterone present in the urine would be expected to form biologically inactive ketosteroids during hydrolysis and extraction. (Likewise, Venning *et al.* in 1942 found that androsterone sulfate is hydrolyzed largely to inactive steroids such as androstenone-17.) Accordingly, the proportion of active androgens in raw rabbits' urine is probably higher than that of the urine extracts but still lower than the androgenic titer of human urine. As an additional check on the techniques, two human urine extracts prepared in the same manner were tested by the chick comb method and were found to contain about nine times as much androgenic activity as the rabbit extracts.

TABLE 2. ANDROGENS AND 17-KETOSTEROIDS IN FEMALE RABBIT URINE EXTRACTS

| Rabbit's age months | Urine volume cc. | Fraction | Androgens as androsterone mg. | 17-ketosteroids mg. | % androgens in extract |
|---|---|---|---|---|---|
| 5 | 340 | Four chromatographed eluates | −0.05 | 2.3 total before chromatography | −2 |
| | | | −0.05 | | −2 |
| | | | 0.02 | | 1 |
| | | | −0.02 | | −1 |
| 8 | 150 | Neutral ketonic | −0.01 | 4.0 | 0 |
| | 300 | Neutral ketonic | −0.01 | 3.9 | 0 |
| | 370 | Neutral ketonic | −0.01 | 2.8 | 0 |
| | 400 | Neutral ketonic | 0.00 | 4.7 | 0 |
| | 360 | Neutral ketonic | 0.03 | 4.5 | 1 |
| 10 | 150 | Neutral ketonic | 0.04 | 1.4 | 3 |
| | 375 | Neutral ketonic | 0.02 | 2.8 | 1 |
| | 1000 | Neutral ketonic | 0.03 | 8.0 | 0 |
| | 390 | Crude neutral | 0.00 | 2.5 | 0 |
| | 500 | Crude neutral | −0.01 | 3.3 | 0 |
| (Average female) | 390 | Neutral ketonic | 0.01 | 4.0 | 1 |

TABLE 3. RECOVERY OF ANDROSTERONE IN RABBIT URINE EXTRACTS

| Androsterone mg. | Added to | 17-ketosteroids in unspiked aliquot mg. | 17-ketosteroids in spiked aliquot mg. | Androgens in unspiked aliquot mg. | Androgens in spiked aliquot mg. | Recovery of 17-ketosteroids % | Recovery of androsterone % |
|---|---|---|---|---|---|---|---|
| 3.6 | Neutral ketonic fraction | 5.0 | 8.6 | 0.03 | 3.69 | — | 103 |
| 1.4 | Neutral ketonic fraction | 4.1 | 5.5 | 0.02 | 1.32 | — | 93 |
| 1.4 | Neutral ketonic fraction | 3.5 | 4.9 | 0.01 | 1.42 | — | 99 |
| 1.4 | Neutral ketonic fraction | 4.1 | 5.5 | 0.02 | 1.52 | — | 116 |
| 3.1 | Urine before hydrolysis | 2.8 | 6.0 | 0.04 | 1.35 | 102 | 42 |
| 3.1 | Urine before hydrolysis | 2.6 | 5.5 | 0.00 | 1.07 | 94 | 34 |
| 3.1 | Urine before hydrolysis | 3.2 | 5.7 | 0.12 | 1.05 | 82 | 30 |

Since the beta-ketosteroid fraction was not present in detectable amounts (Table 4), neither dehydroisoandrosterone nor isoandrosterone apparently are excreted in appreciable quantities by normal rabbits. (Similarly, no digitonin-precipitable ketosteroids were found in normal guinea pig urine by Miller and Dorfman, 1945.)

The proportions of alcoholic and non-alcoholic ketosteroids (Table 4) do not represent those originally present in the urine since acid

TABLE 4. ALPHA & BETA, OH & NON-OH KETOSTEROIDS IN MALE RABBIT URINE EXTRACTS

| Rabbit's age months | Urine volume cc. | Alpha-ketosteroids mg. | Beta-ketosteroids mg. | Alcoholic ketosteroids mg. | Non-alcoholic ketosteroids mg. |
|---|---|---|---|---|---|
| 7 | 470 | 2.9 | 0 | — | — |
| | 535 | 2.9 | 0 | — | — |
| | 525 | — | — | 0.89 | 0.63 |
| 12 | 255 | 3.2 | 0 | — | — |
| | 300 | 3.6 | 0 | — | — |
| | 225 | — | — | 0.68 | 0.75 |
| 16 | 470 | 2.9 | 0 | — | — |
| | 500 | 2.1 | 0 | — | — |
| | 400 | — | — | 0.78 | 0.66 |

hydrolysis causes a large conversion of alcoholic ketosteroids to non-alcoholic ketosteroids (Pincus and Pearlman, 1941).

The exact chemical nature of the neutral 17-ketosteroids excreted by rabbits must await their separation and identification.

## SUMMARY

Low androgenic titers are found in rabbit urine extracts. In terms of androsterone, only about 2% of the neutral ketonic fraction (less than 1% of female) rabbit urine is biologically active. Recovery experiments and comparative data indicate that the proportion of androgens in untreated rabbits' urine, while probably considerably

# NOTES ON THE THIRTIETH ANNUAL MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting was held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

Forty-seven papers were presented and forty-four papers were read by title. Total registration was 458, equally divided between members and non-members. Two hundred, twenty-two were present at the dinner at which the presidential address was given by Doctor C. N. H. Long.

Actions of general interest taken by the Council are as follows:

(1) It was the decision of the Council to hold a Postgraduate Assembly in Oklahoma City, February 21–26, 1949, similar to the one which was so well received in Los Angeles in 1948.

(2) The Council approved the indexing and publication of ENDOCRINOLOGY, Volumes 1 to 40, inclusive. These will be published by Mr. Charles C Thomas, in two editions, one including the indices of Volumes 1 to 25, and the other, Volumes 26 to 40.

(3) It was the Council's decision to publish the transactions of the American Goitre Association.

(4) The Council accepted with much regret the resignation of Doctor Earl T. Engle, Chairman of the Publications Committee, and appointed Doctor Warren O. Nelson to this office. Appreciation was expressed to Doctor Engle for his work as chairman of this committee.

(5) The Committee on Registry of Endocrine Pathology was re-appointed to negotiate with the Scientific Director of the American Registry of Pathology regarding the formation of an Endocrine Registry at the Army Institute of Pathology. A sum not to exceed $750.00 was appropriated for necessary expense for one year.

(6) It was voted to appropriate $250.00 for 1949 to the National Society for Medical Research.

(7) Due to increasing cost of labor, paper and all printing materials, and the enlargement of the Journals, the dues were increased to $11.00 per year which includes subscription to either of the Journals, with a combination offer of $16.00 for both Journals.

(8) The Council voted that the thirty-first Annual Meeting be held June 3 and 4, 1949, in Atlantic City, New Jersey.

(9) A list of the 1948–49 Officers, Council and Committees follows:

*Officers*

| | |
|---|---|
| President | John S. L. Browne |
| President-Elect | Edward A. Doisy |
| Vice President | James H. Means |
| Secretary-Treasurer | Henry H. Turner |

*Council*

| | |
|---|---|
| Frank A. Hartman | Roy G. Hoskins |
| Carl R. Moore | E. Perry McCullagh |
| Gregory Pincus | Fuller Albright |
| C. N. H. Long | Mayo Soley |
| Paul Starr | John S. L. Browne |
| Edward A. Doisy | James H. Means |

Henry H. Turner

*Publications Committee*

W. O. Nelson, Chairman
Roy Hoskins
E. Perry McCullagh

*Awards Committee*

P. E. Smith, Chairman
E. C. Kendall
F. D. Lukens
Edward A. Doisy
Lawson Wilkins

*Nominating Committee*

John E. Howard, Chairman
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Leo T. Samuels

*Committee on Registry of Endocrine Pathology*

E. W. Dempsey, Chairman
Evelyn Anderson
W. U. Gardner

*Membership Committee*

C. N. H. Long, Chairman
George W. Thorn
Willard O. Thompson

*Committee on Postgraduate Course in Endocrinology*

Henry H. Turner, Chairman
John S. L. Browne
Lawson Wilkins

*Committee on Nomenclature*

Elmer Sevringhaus, Chairman
Hans Selye
Thomas McGavack

*Consultant Committee to Food and Drug Administration*

A. Kenyon, Chairman
George Sayers
J. P. Pratt

# ASSOCIATION AWARDS FOR 1949

## THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

## THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

## THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

# NEW BOOK

General Endocrinology. By C. Donnell Turner. Published by the W. B. Saunders Co., Philadelphia, Pa., 1948. v+604 pp. Price $6.75.

The concept of internal secretion, classically attributed to Claude Bernard, has found application in many physiological situations and has been amplified to include a multitude of substances which collectively comprise the chemical regulatory system of living organisms. A part of this regulatory system concerns the ductless glands, the study of which is the subject material of Endocrinology. Dr. Turner's book presents the basic and fundamental information of this latter field.

The point of view of the biologist rather than that of the physician has been emphasized throughout. The effort is to understand how systems work rather than how symptoms develop. Clinical information is not unduly slighted, but is placed in perspective with experimental observations and used insofar as it is of value in elucidating the way the endocrine system operates.

In any such presentation, an author has a hard time drawing the arbitrary line between the proper hormones and the closely related chemical regulators. Dr. Turner is no exception: he has included chapters on the plant hormones and the gastrointestinal principles but has treated cavalierly subjects such as acetylcholine, glucose and amino acids. This comment is not meant as a criticism, but indicates the difficulty one has in drawing sharp lines in endocrinology.

The author has chosen to present the basic and established facts and does not wander too far into the territory of controversial issues. This is undoubtedly a wise choice, particularly since the book was designed as a text for college students in biology and not for the more advanced students in professional schools. Nevertheless, the final result is a book somewhat more elementary than that ideally designed for medical students and physicians. The elementary student undoubtedly needs facts with which to think, and the elementary textbook should contain the agreed-upon factual background of the subject. For the more sophisticated student, on the other hand, training in how to think is advisable and perhaps no better method exists than to provide examples of how masters have thought and are thinking their way through controversial issues. Admirable as Dr. Turner's book is, it needs to be supplemented by another more advanced account for the more mature student.

These reflections concern matters related to the pedagogy of endocrinology as a whole, rather than the present book under review. Dr. Turner has defined the scope of his effort accurately and has acquitted himself excellently. The book is attractively printed and bound, contains a useful index and many informative drawings for which it was written, and it will find usefulness in many other areas as well.

# ENDOCRINOLOGY

VOLUME 44 FEBRUARY, 1949 NUMBER 2

# A TECHNIQUE OF ORGAN CULTURE FOR PROTRACTED METABOLISM STUDIES

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THE DEVELOPMENT of a perfusion apparatus capable of sustaining life and growth in an excised organ has occasioned much study. Carrel and Lindbergh (1938) presented the technique as developed by them and their associates at that time. Most recently Long (1946) and Anderson and Long (1947) and Hetcher (1948) have shown the value of an in vitro study of organ function by this type of technique.

None of the above workers has been able to state that growth was grossly visible in their preparations. In their discussion Carrel and Lindbergh point out that the absence of red cells in the perfusion medium probably kept their preparations from exhibiting full function. The work of Anderson and Long has been concerned chiefly with short time studies in which growth would be difficult to ascertain.

Our work has dealt with repeated studies on the same isolated organ perfused for several days. Each single study lasted approximately 24 hours. We felt that the data obtained would not be of real significance unless we could be certain that under the conditions imposed the organ was capable of showing growth or healing. We have directed the development of the technique toward that end.

Several specimens studied have demonstrated this capacity conclusively. As part of the study biopsies were made on the 2nd and 3rd

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American Medical Association Therapeutics Research Committee Grants No. 565 and 569.

[1] Obtainable from Dewey and Almy Chemical Company, Cambridge, Massachusetts.

day of perfusion. Healing of the wound and closure of the biopsy aperture occurred 72 to 96 hours later. It seemed to us that a description of the technique in fairly extensive detail at this stage of development would be of value to other workers.

## DESCRIPTION

To illustrate the apparatus a working diagram is presented in Figure 1. Figures 2 and 3 show the apparatus used for obtaining an alternating pressure and vacuum, and Figure 4 illustrates the type of cannula employed.

Since Figure 1 is not to scale the following dimensions of importance should be kept in mind.

1. The Erlenmeyer flasks from which the organ chamber and fluid reservoir are made are of 1000 c.c. capacity.
2. The pyrex tubing used is of heavy wall $\frac{1}{4}''$ internal diameter.
3. The rubber tubing has a bore of $\frac{1}{4}''$ with a wall thickness of 3/16″. Only pure gum, autoclavable tubing is employed.
4. The rubber diaphragm (1) used in the pulsator tube (2) consists of the neck of a stratosphere balloon.[1] The function of the rubber diaphragm is solely to separate the perfusion fluid and the pulsating air. The apparatus could be operated without the use of the diaphragm but then a sterilizing bulb such as Lindbergh employed would have to be placed in the pulsating air column.
5. The valves do not have ground glass contact surfaces. They are highly polished to provide firm closure. The high polish reduces to a minimum the grinding and destruction of red cells.
6. The "rubber bushings" (3) used are of the "Fenwall" type. One closes the pulsator tube (2) and the other the organ chamber. Sufficient flexibility is provided by the latter to permit the arranging of the organ chamber in relation to the organ so that the organ can rest on the side of the chamber without undue stretching of the artery. The need for such flexibility is found essential in mounts with extremely small arteries. If torsion or jamming of the artery over the mouth of the cannula occurs, the rate of blood flow with the same activating pressure can be reduced as much as 80% of that possible with a clear passage.
7. The tapered joints used on the connector (14) are standard taper 10/30.
8. The organ chamber has been used in many shapes. The tube shown is quite generally useful for small organs. For large organs we use a chamber from which the large end opposite the cannula has been blown out and a ridged rim fashioned around it. An opening large enough to insert the hand can be provided when the flask is a 1000 c.c. Erlenmeyer. These large chambers are closed by fitting a surgeon's glove over the open end so that the wrist portion fits over the rim and the fingers project into the interior. The hand can then be

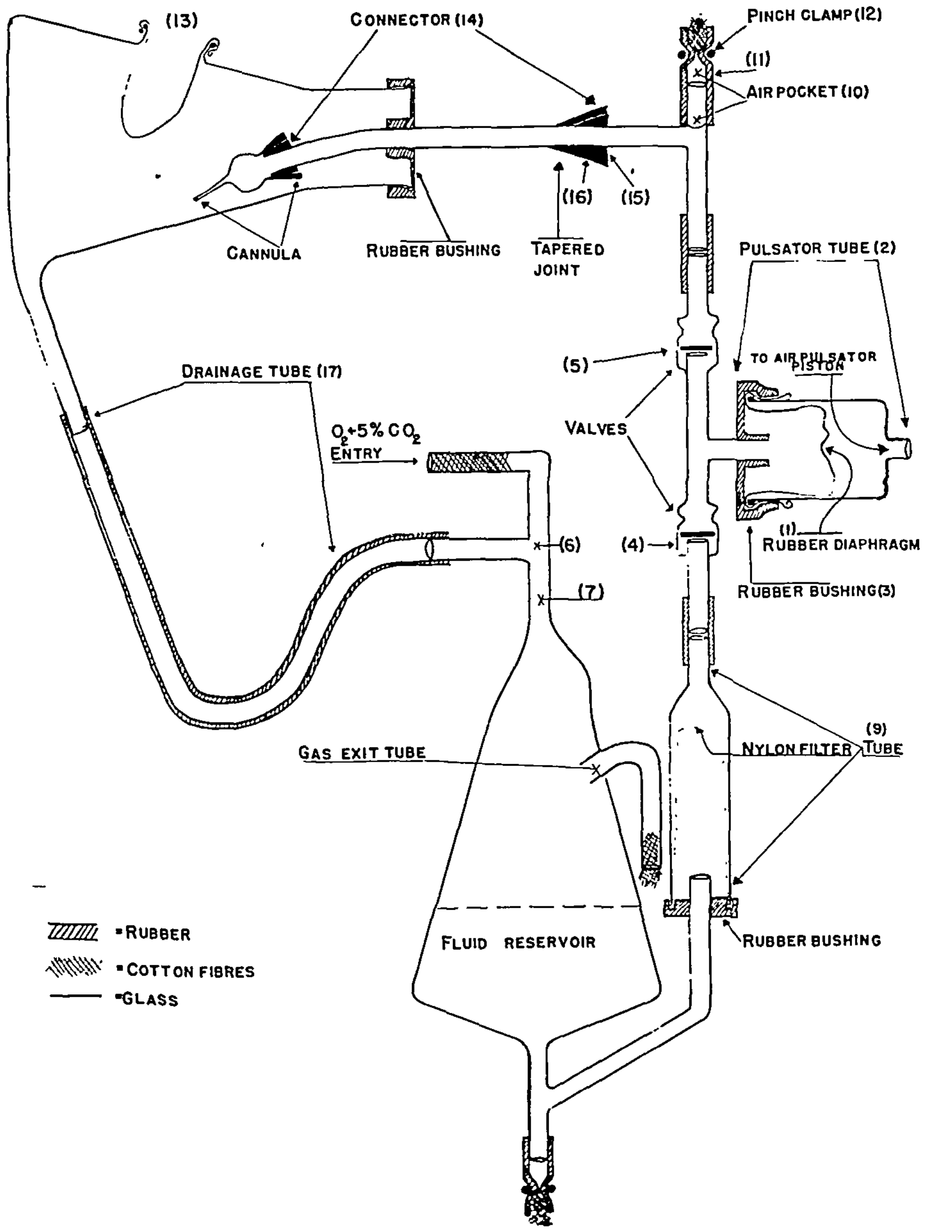


FIG. 1. Schematic diagram showing arrangement of parts of perfusion pump. Legend indicating nature of material is applicable throughout diagram except where so labeled in pulsator tube portion (2) and at the orifice (13) of the organ chamber. At *these two points the single fine line indicates the use of a rubber condom or balloon neck* as a flexible rubber diaphragm.

inserted into the glove and the organ and arteries palpated during a perfusion.

## OPERATION

As can be seen from Figure 1 the two valves are so arranged that the fluids can circulate in only one direction. The lower valve (4) serves to prevent fluid from returning to the reservoir on the pressure stroke. The upper valve (5) which rises on the pressure stroke, holds the residual pressure during the pump's negative stroke and refilling of the rubber diaphragm. The fluid is forced into the artery through the cannula and emerges from the veins of the preparation.

As the organ chamber and blood reservoir are both at atmospheric pressure, the perfusate flows freely down the walls of the chamber and into the duct leading to the fluid reservoir.

Just prior to entry into the organ chamber the blood passes the mouth of the gas entry tube (6). The gas and blood flow down tube (7) into the organ chamber. Oxygenation and $Co_2$ removal from the fluid occur at this time. The process is usually complete by the time the fluid reaches the well of the fluid reservoir. The gas passes out of the chamber through the exit (8). A mixture of 5% $CO_2$ in Oxygen has been found requisite to maintain the pH at 7.2.

Since small bits of tissue, blood clots, etc. could cause a complete stoppage of flow, the blood is filtered in tube (9). The filter is a very fine mesh nylon bag.

The diaphragm chamber has a total volume of about 100 c.c. Of this volume about 50 c.c. is occupied by fluid within the diaphragm. The total volume of air being compressed is about 1000 c.c. Under the conditions of operation about 10 c.c. of fluid passes through the organ at each beat under maximum flow conditions and 0.02 at a minimum. Therefore, with a varying rate of blood flow the applied pressure on the organ can not change appreciably even though the change from minimum to maximum occurs within a few minutes.

The diastolic pressure is maintained by the air trapped at point (10). The volume of this air can be adjusted by introducing it with a sterile hypodermic needle through the rubber closure (11), or by allowing it to escape past the pinch cock (12).

The actual pressures are determined by a simple compression device inserted through the aperture (13). The device consists of a glass tube with a rubber diaphragm tied over one end. Fused to the tube is a glass rod which is bent in such fashion that expansion of the diaphragm causes it to push against the rod. The artery is placed between the diaphragm and the rod. The artery extending from the device to the organ is palpated by inserting fingers through the rubber glove previously mentioned as closing the end of the largest chambers. Pressure is exerted and measured by the usual sphygnomometer arrangement. The points of maintained turgor of the artery and the

onset of pulsation mark the diastolic and systolic pressure levels respectively. These observations, while accurate enough to demonstrate the tremendous pressure loss induced by the cannula orifice, are too inaccurate for satisfactory study of the flow problems involved.

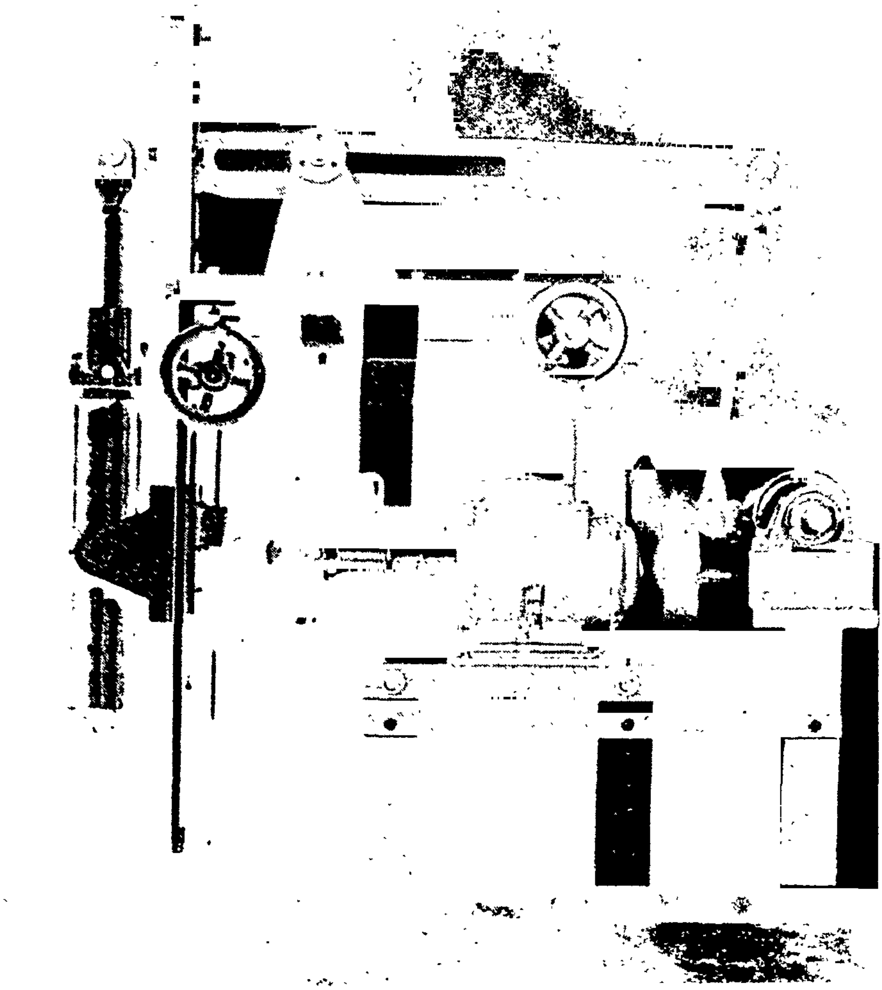

FIG. 2. Photograph of mechanical portion of actuating mechanism.

Efforts are being made to solve the problem of pressure measurement by electrical means.

## BLOOD PRESSURE CONSIDERATIONS

In the work done previously blood pressure has often been measured in the glass portion of the apparatus. This type of determination was a feature of both the Long and Hechter and the Carrel and Lind-

bergh instruments. Under certain circumstances, such as when the arteries cannulated are large, this leads to but small error in the determination. When, however, the rate of flow thru the cannula is large so that friction loss is great, and when, as we have found preferable, the subsidiary arteries are not ligated, then a determination of the pressure anywhere but on the artery is relatively meaningless. It is obvious that under these circumstances that the actuating pressure must be very high to maintain 100 mm. of Hg pressure or more in the organ. It is for this reason that our apparatus has such a large capacity.

## DRIVING MECHANISM

In Figures 3 and 4 are shown a photograph and diagram of the assembled apparatus illustrating the principles of operation.

This mechanism meets the following specifications:

1. It can deliver to the pulsator tube described earlier (No. 2 Figure 1). from 0 to 30 lbs. per sq. in. positive pressure and from 0 to 5 lbs. negative pressure on the opposite stroke of the piston.
2. Within requisite limits the positive and negative pressures can be adjusted independently of one another.
3. The rate of pulsation can be altered from 1 beat per minute to 150.

The amount of compression is regulated by moving the fulcrum point (1) Figure 3 along the slot (2) of the rocker arm (3). The amount of vacuum can be altered by either of two ways. First, by moving the cylinder (4) to a new position relative to the piston (5). This action increases or decreases the amount of "overshoot" of the piston in relation to its starting position. Secondly, air can be allowed to escape slowly from the system on the compression stroke. When the negative pressure on the return stroke equals that of the column of water in tube (7) air will again enter the system by bubbling up from the bottom of tube (8). The vacuum level can be further adjusted by regulating the height of the water column.

The apparatus was actuated by a $\frac{1}{2}$ horsepower motor driving the apparatus thru an adjustable gear ratio. The need for the massive construction is due to the fact that at 15 lbs. per sq. in. operating pressure the total thrust on the piston rod exceeds 400 lbs.

The handwheels shown in the photograph regulate the position of the walking beam fulcrum and the cylinder. Duplication of the settings for different experiments is facilitated by dials (not visible in photograph) mounted on the hub of the wheel.

We have found it requisite to use the water column arrangement continuously to maintain the setting of the apparatus. The most minute leaks will in time permit the loss of much air and so cause a drop in the actuating pressure and increment in the vacuum. The system as built works very effectively and its simplicity of construction and maintenance is a desirable feature.

In our earlier work, before developed the driving mechanism described above, we employed the method described by Carrel and Lindbergh and modified by Hechter. It was found to be adequate for the pressures they utilized but could not function adequately in the range which we found necessary for the reasons discussed above. When the

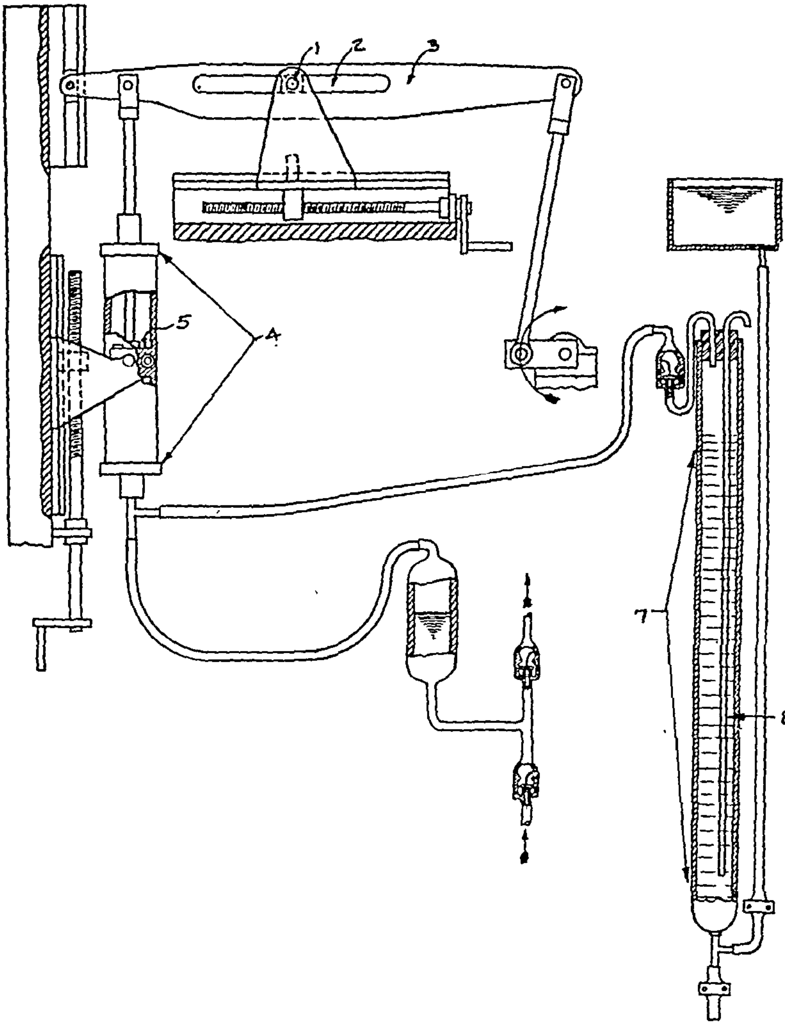


Fig. 3. Working diagram of actuating mechanism showing also relation to pump of "inverted" water column used to regulate vacuum and compressed air volume.

operating pressure in the fluid line had to be raised to 15 lbs. per sq. in. we found difficulty in obtaining surges in the oil vessel of an adequate intensity to give a sharp pulsation. Furthermore the amount of compressed air consumed at these operating pressures was enormous and called for very heavy duty compressors as a source of the operating pressure. While the initial expense of the apparatus described

is higher than that of the mechanical portion of the Carrel Lindbergh design or of Hechter's modification we have found that the greater reliability and ease of adjustment at the pressures we utilize makes the latter design mandatory for the long term perfusions.

## STERILITY AND MOUNTING OF PREPARATIONS

Absolute asepsis, as pointed out by Carrel, is an essential of a successful prolonged perfusion. The elaborateness of the technique for prevention of infection must, of necessity, vary from laboratory to laboratory and is dependent on the conditions found in each institution.[2] We have found the use of glass shields between the operator and the specimen, a steam filled culture room, liberal application of chlorazene and alcohol to surfaces and instruments, adequate for success. Infections in the animals used as donors for blood and organs have proven more of a problem than technical failure.

As can be seen, the organ and culture fluid are not exposed to contamination once placed in the pump. If fluid is drained from the reservoir, the drain point can be sterilized again by washing out the tube with cotton soaked in a suitable germicide. Clamping of the tube and drying of the cotton prevent infection prior to the next draining.

New fluids and solutions of specific materials for study can be quantitatively introduced by injection through the thick walls of the rubber tubing employed. "Arterial-venous" differences in concentration may be ascertained by withdrawing samples of the perfusing fluid at the appropriate points in the cycle.

The operations involved in setting up the pump vary with the different organs and animals used as donors. In general we followed the technique described below:

1. Sodium sulphide is used as a depilatory. The skin is then washed thoroughly with hot water and 70% alcohol. Sterile towels are draped over those portions of the animal which have not been treated with the sulphide. The usual precautions for aseptic surgery are then followed.
2. After excision, the organ is placed in a large petri dish containing Tyrodes solution plus 10% by volume of a dextrose and sodium citrate solution.[3] The citrate solution is added to prevent clotting of the blood in the exposed blood vessels.
3. The cannula is inserted into the artery.
4. The artery is tied on to the cannula with number 00 surgical

[2] Carrel describes an elaborate procedure for asepsis. In our experience such elaborateness is requisite in dusty localities such as cities. Conversely in suburban or country regions few precautions are requisite. The work described here has been done in a suburb of Worcester. In the earlier stages of the development of this technique (1937 et seq.) while working in urban communities we too found an elaborate technique obligatory.

[3] Composition = 13.3 grams sodium citrate, 4.7 grams citric acid, and 30 grams dextrose, per liter.

silk thread. The loose ends of the tie are brought up over the bulge in the cannula which begins at the end of the grinding (Figure 4). At this point each end of the thread is tied to the cannula by a clove hitch. Tightening of this hitch forces the tie around the artery up the cannula until it is jammed against the increasing taper. Since the

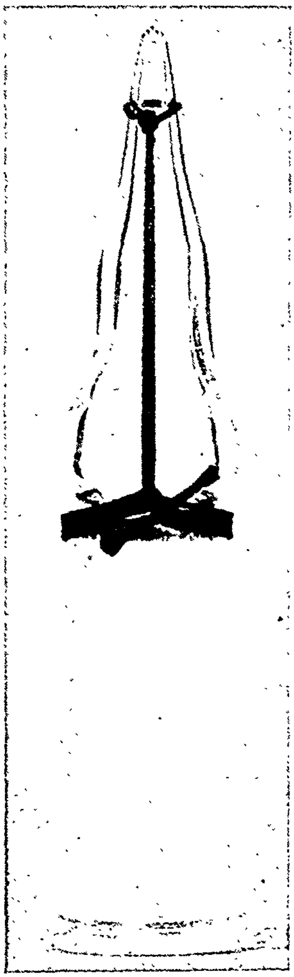

FIG. 4. Type of cannula inserted into artery of perfused organ. Manner of tying artery in position as described in text is also illustrated.

clove hitch knot will not stay tight permanently on a smooth surface the ends of the two threads are again passed around the cannula and knotted.

This combination of ties over (1) the tapered portion of the cannula and (2) the bulge makes certain that the artery will not slip off. The thread running from the bulge to the artery also serves as

an index of which side of the cannula should remain uppermost. While it is easy to see a twist in a large blood vessel, one can not always be certain of avoiding this difficulty when small vessels are employed.

5. After cannulation, the artery is flushed with dextrose citrate and Tyrodes solution to remove blood.

6. It is then attached to the connector marked (14) in Figure 1.

7. The organ is placed in the organ chamber through the mouth and the Fenwall stopper snapped into position.

8. The assembled unit is then carried to the incubator where the male joint (15) is inserted into the female (16) and the drain tube (17) connected to the blood return tube of the blood reservoir.

9. By alternately blowing and sucking on the tube leading to the compression chamber, the perfusion fluid is forced past the valves and up to the base of the air trapped at point (10).

10. The pump is then started on a pressure stroke.

We do not find that air emboli constitute a problem.

Only cannulated vessels of the size of the rabbits' femoral artery need to be tied. All the subsidiary arteries can be left open. Naturally such "leaks" increase the rate of blood flow so that settling out of the red cells does not occur. They also make easy the removal of air from the specimen if any is trapped. (In practise they also serve another purpose, that of estimation of the blood pressure in the perfusion of organs whose arteries are too small to permit palpation.). Obviously "hemorrhage" from an artery should squirt out if the blood pressure were normal. The height of the squirt is a function of the pressure. The fact that squirting occurs does not, of course, reduce the blood pressure to those branches of the cannulated artery which lead to the tissue being perfused. Connective tissue frequently overgrows these open ends, but a touch usually dislodges the overgrowth.

## PERFUSING FLUIDS

It was found necessary to use red cells to obtain growth. Between 0.5 and 2 million per c.c. appear adequate. Blood is now drawn from the donor animal into a Fenwal flask and kept from clotting by using the citrate solution in a ratio of 1 c.c. citrate to 5 c.c. blood. The mixture is then diluted by half with White's solution and if clotting does not occur, calcium chloride crystals are added until clotting takes place.

The clots which form are broken up by shaking. Filtration of the blood through the standard nylon bag arrangement removes the clot fragments. The needle at the end of the filtering set-up (see below) is inserted into the drain tube from the organ chamber. This arrangement permits the filtering of the perfusing fluid and filling of the fluid reservoir at the same time.

At first we tried to use whole blood after clots had been removed.

All too frequently after several hours (10–12) of perfusion we found the blood beginning to clot again.

Our attention being at one point turned from animal organs to human organs, we were led to the technique finally devised. The blood for these perfusions could most expeditiously be obtained from a blood bank. Such blood contained 25% by volume of the citrate and dextrose solution mentioned previously. After inducing clot formation by adding $CaCl_2$ to 500 c.c. of citrated human blood, 250 c.c. could be drawn off. To increase our supply it seemed expedient to dilute the blood and Tyrodes solution was first employed. We did not find growth in perfusions set up with it as a diluent.

White's solution[4] (1946) was next used as diluent. It was found immediately that more $CaCl_2$ had to be added to such a mixture to induce clot formation than when Tyrodes had been used as diluent. However, once the mixture had clotted no further clots formed during a perfusion. It was with this mixture that growth was obtained. Six hundred to 700 c.c. of perfusion fluid containing 1 to 2 million red cells per c.c. became available after filtering off the clot when 400 c.c. of blood was diluted by 500 c.c. of White's solution and 100 c.c. of the dextrose citrate solution.

Obviously a solution providing less than 50% of the normal colloid osmotic pressure and only 1 million red cells per c.c. could stand improvement. Further work should be done to determine the amount of protein requisite to obtain maximum response. Good methods for the liberation of red cells from the clot are also needed.

## HANDLING OF THE SOLUTIONS

The volume of sterile fluid employed in this technique is far larger than is generally used in tissue culture techniques. To expedite the process certain methods worthy of mention have been worked out.

We have found that the blood bank equipment designed for the "Fenwal System" is extremely convenient. This system centers around the use of a flask with a large mouth. Thru the use of a stopper such as we employ in the pump described earlier, various closures of the sterilized flask are available. One of these permits introduction and removal of fluid by hypodermic needles. We have found this closure extremely helpful. Use of a double needle permits filtration under vacuum of White's solution when large quantities (500 c.c.) are to be filtered. For smaller volumes, the vacuum present in the flask after autoclaving suffices. Only pyrex utrafilters are used.

Since we do not use Tyrodes solution alone in our work and always in an atmosphere of 5% $CO_2$ we employ a simplified method of sterili-

[4] White's solution is a completely synthetic medium capable of maintaining growth of various tissues for 1 to 2 weeks. It contains all the essential amino acids, glucose and vitamins found requisite by White in his remarkable study. We employ it as described by him except that we do not add the phenol red he employs as a pH indicator.

zation which involves autoclaving of the bicarbonate fraction. Such autoclaving is not ordinary practise as loss of $CO_2$ and an increment in pH results.

The solution was made up in 3 parts—A, B, and C as listed below.[5] Two test tubes of 20 c.c. volume were placed in the Fenwal flask. Into these were pippetted 10 c.c. each of solutions B and C. The largest volume (180 c.c.) containing 10 c.c. of A and 170 c.c. $H_2O$ is placed in the body of the flask.

By keeping the flasks vertical during autoclaving and storage the 3 solutions are prevented from mixing. Tilting the flask several times before use mixes the solutions and a "fresh" solution of Tyrodes is available. This method of preparation permits the manufacture and storage of the solution in bulk quantities for prolonged periods. This is a tremendous advantage as occasionally the changing and washing of a pump for a new experiment on an organ involves the use of as much as 1 to 1.5 liters of Tyrodes solution.

Transfer of fluids from containers into the pump is made by the use of the drip technique employed in blood transfusions. When the mixture of White's and blood was transferred to the pump a nylon filter similar to the one employed in the pump is inserted into the line. Such prefiltering reduces the load on the filter in the pump and increases the time during which the filter can function.

## DAILY ROUTINE OF ORGAN MAINTENANCE

The fresh perfusion fluid is removed from storage and placed in the incubator to reach the proper temperature.

A sample of the perfusing fluid is removed from the pump by hypodermic syringe and examined for bacteria.

If bacteria are seen or suspected 20 milligrams of streptomycin and 100,000 units of penicillin are added to the perfusion fluid. Experience indicates that in many instances this action stops bacterial growth.

If no bacteria are found the pump is drained, Tyrodes run in and pumped thru the organ. Several flushings are made in this fashion and the preparation is ready for another experiment. The changeover takes about 15 minutes and it is not requisite to interrupt operation during that period, altho cessation for a short period permits more accurate withdrawal of the perfusate.

If bacteria are found present the organ is installed in a new pump after the surface of the organ has been washed thoroughly with Tyrodes solution containing streptomycin and penicillin. After reinstala-

[5] *Solution A 10 c.c.*

| | |
|---|---|
| NaCl | 160.0 grams per liter |
| KCl | 4.0 grams per liter |
| $CaCl_2$ | 4.0 grams per liter |
| $MgCl_2.6H_2O$ | 2.0 grams per liter |
| $NaH_2PO_4H_2O$ | 1.0 grams per liter |

*Solution B 10 c.c.*

| | |
|---|---|
| $NaHCO^3$ | 20.0 rams per liter |

*Solution C 10 c.c.*

| | |
|---|---|
| Glucose | 20.0 grams per liter |

tion the antibiotics are added to the perfusate and the operation continued for 24 hours.

We find that in order to maintain the organs in good condition the perfusing fluid ought to be changed every 24 hours. At least 200 c.c. of medium is requisite to last thru this period.

To those acquainted with Carrel's work it may appear wasteful to change the fluid in the pump every 24 hours. His data indicated that the perfusion medium could be used for several days with the pH dropping as low as 6.9. We have not yet had the opportunity to study this in detail, as we were more interested in metabolic studies, than in organ survival under adverse conditions. A 24 hour period of perfusion was convenient for our work.

No studies have as yet been made on the rate of removal of the amino acids etc. present in White's solution. Such work has for the present been outside our province.

The gross changes visible in the circulating medium are, however, sufficiently marked to indicate the need for frequent alteration. A scum similar in appearance to that seen on infected serum frequently shows up after 24 hours of perfusion in perfectly sterile preparations. At the end of 72 hours of perfusion it is usually found necessary to transfer the organ to another pump because of filter blockage and inability to wash out the "scum" stuck to the walls of the fluid reservoir.

In several instances it has been possible to see gross changes in the organ when the fluid has been changed, brighter color, better blood flow, etc. One remarkable response has been an extreme contraction of the psoas muscle without other stimulation on the 4th day of a perfusion.

Therefore, while we have no concrete evidence at the moment as to the need of frequent change of perfusate, it is our sentiment that such work will provide data indicating the need for addition of food material and removal of waste products at frequent intervals.

## OBSERVATIONS ON ORGAN VIABILITY AND NORMALITY OF FUNCTION

As mentioned earlier, our primary interest in the development of the technique described above has been the furtherance of the study of the metabolism of the steroid hormones. These studies have received our primary attention and we have neglected completely to make any studies of the protein, glucose, amino acid or vitamin metabolism under the imposed conditions.

We have had the problem, however, of validating our findings on the steroid hormone metabolism in the sense that experiments done on dead or dying tissue would be of small value in comprehending the normal situation. It appeared to us that growth of the organs, particularly in the cut portions, would be an adequate criterion of success in life maintenance.

Such growth is difficult if not impossible to describe for publication unless periodic photographs of the organ are taken during the experiment. It will not be further elaborated upon here except to point out that in several instances where the perfusion has lasted for sufficient time, almost complete healing of cut surfaces has occurred. In one perfusion of a human skin rope graft the connective tissue growth in 48 hours was such that at least two cubic centimeters of new tissue was formed around the cannulated blood vessel. Since no bleeding occurred at that point nourishment of the growing tissue could only only have come from a functioning vascular bed.

We have adopted the practise more recently when working with human tissue to remove a portion for section prior to the perfusion and comparing it with a specimen obtained at the end of the run. This technique is only of value when the experiment is terminated without an infection having entered the system. Very short exposures to an infection spoil the experimental specimen.

Figures 5 and 6, show the histological appearance of a human ovary and human muscle before and after perfusion. The muscle was fixed after a heavy infection had occurred. Twenty-four hours

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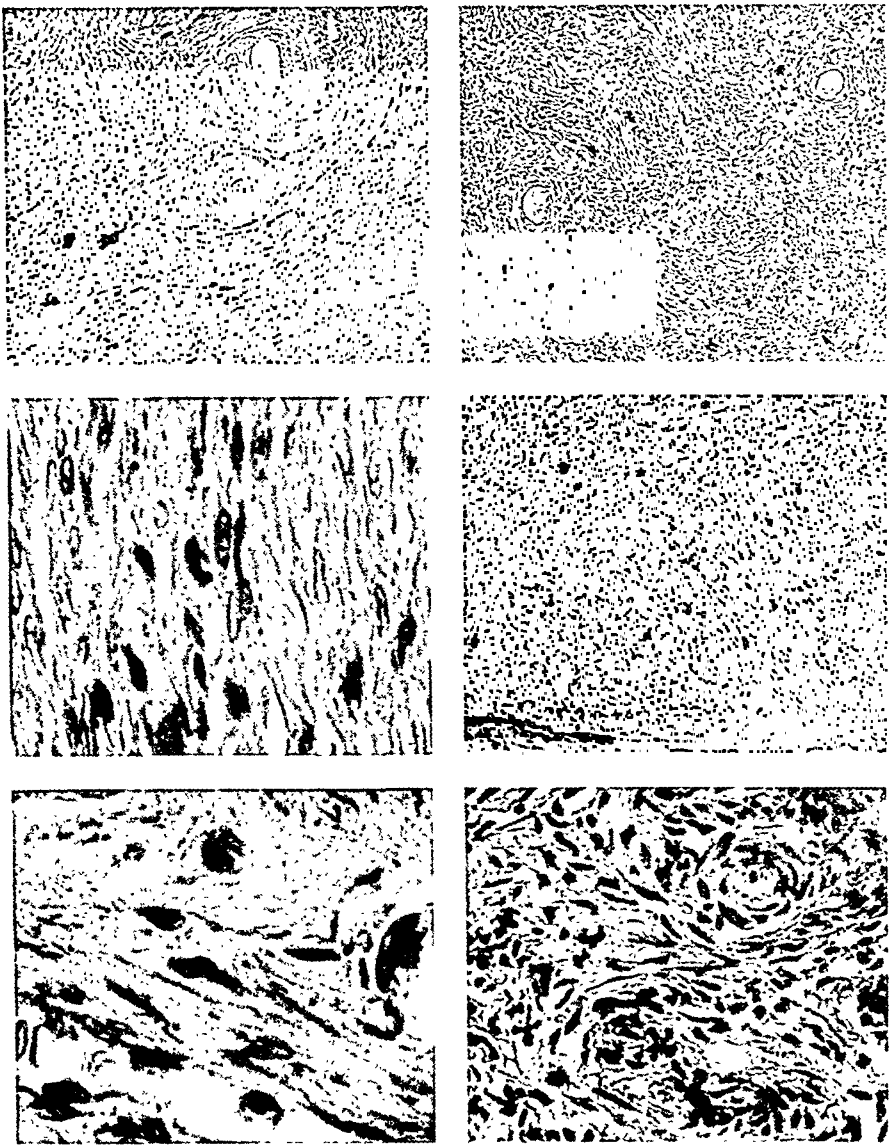
FIGURE 5

*Control, Human Ovary.* Four sections representing ovary, tube and fibro-fatty tissue. The ovary reveals one moderately sized Graffian follicle containing a small amount of blood and detritus in the lumen. The lining is broken in several places but is made up of several layers of large cells arranged in moderately compact fashion. The cells themselves have ovoid to circular nuclei containing a moderate amount of nucleoplasm. The cytoplasm is coarsely reticular and in places granular. This represents the theca interna. The theca externa shows a layer of fusiform cells resembling fibroblasts and having elongated fusoid nuclei devoid of definite nucleoplasm. They give the appearance of being compressed because of their form and position. Fibrillae are present but not abundant. There is a large network of capillaries in this region. The remaining ovarian tissue shows a fair amount of stroma made up of the normal fusiform cells and fibrous connective tissue. The blood vessels are abundant and thick walled. The section of tube shows a mucosa which has lost its normal pattern. The interlacing branching formations are considerably reduced. Here the formations are thickened with fibrous connective tissue surrounded by low columnar and in places ciliated epithelium. No stratification is seen. There are areas which appear to be sneered off by the fibrous connective tissue preservation and this has greatly reduced the size of the lumen. These sneered off areas have large irregular acini-like spaces with broken thin partitions dividing them. The remaining tube wall is not remarkable, except for the great number of small thick-walled blood vessels and fibro-fatty tissue indicative of old chronic inflammation. Exudative cells are absent.

*Experimental, Human Ovary.* These sections of ovary show for the most part fairly good preservation of normal cellular architecture. There is moderate edema throughout. One involuting luteal cyst present. This has a peculiar amorphous architecture with columnar and pseudo-columns of large lipoid-laden cells either devoid of nuclei or having small deeply staining irregular nuclei. The cytoplasm when present is for the most part reticular or granular. In some of these areas center cores of fibrous connective tissue can be seen. The surrounding tissue is fibrous in character. The remaining ovarian tissue shows irregular, for the most part elongated, fusiform and in places wavy nuclei. There are many fibrillae but the edema renders the stroma less dense than normal and everywhere one encounters small nuclei suggestive of degeneration and necrosis. The blood vessels are thick-walled and fairly well preserved. Some contain agglutinated and conglutinated blood. Thrombosis is not a prominent feature.

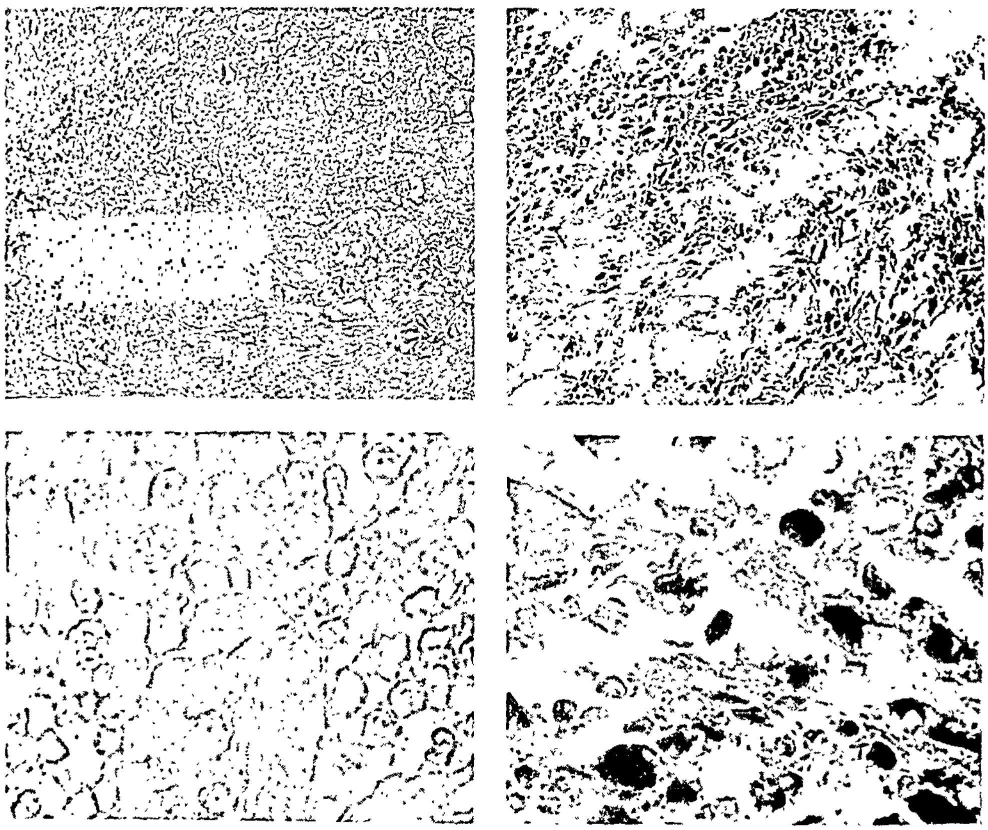


CONTROL TISSUE HUMAN OVARY EXPERIMENTAL TISSUE

FIG. 5. Infection found 6 hours before taking specimen. Perfused 120 hours. During 3rd. day of operation cannula and organ were blocked by clot formation. This was released by forcing Dextrose Citric Acid solution thru organ. Sheep Pituitary Gonadotrophin (500 R.U.) was added to perfusate on 4th day, (see table). Several areas of ovary showed infarcts on gross examination indicating incomplete perfusion. (Pathologist's description of tissue is shown on the facing page.)

previous to taking the specimen our notations on the latter experiment indicate a continuing growth of connective tissue. Appended to each of the photomicrographs is a description of the tissue as pre-

CONTROL TISSUE



PERFUSED TISSUE
HUMAN MUSCLE

FIG. 6. Human muscle taken from near tumor site. Heavy infection found in perfusate 12 hours prior to biopsy. Perfused 96 hours. (Pathologist's description is shown on next page.)

pared by Dr. Kaneb. These descriptions tend to indicate a decrement in the viability and general health of the specimen after several days perfusion even in the ovary. It must be recognised, however, that in each instance the perfusion was halted because of the presence of

bacteria in the perfusate. Consequently toxic materials were present. Considering, however, that toxins, "anemia," and inadequate protein concentration in the perfusing fluid all tend to reduce the probability of viability, it is indeed surprising that the specimens can look as well as the ovary shown.

Physiological evidence of function is available in the response of ovaries to gonadotrophin. We have been able to induce ovulation in cow and rabbit ovaries by the addition of large amounts of gonadotrophin to the perfusate. This has not been observed in human ovaries but here an increment in the estrone concentration of the perfusate has been seen as shown in Table I.

TABLE I

| Ovary | Extraction | Days of Perfusion | Gonadotrophin Added | Total Estrone[6] Concentration in perfusate in micrograms |
|---|---|---|---|---|
| Ovary[7] | 31 | 1 | 0 | 16 |
| | 32 | 2 | 0 | 16 |
| | 33 | 3 | 0 | 21 |
| | 34 | 4 | 500 R. U. | 55 |
| | 50 | 5 | 0 | 26 |
| Ovary | 51 | 4 | 500 R. U. | 36 |
| | 53 | 5 | 0 | 26 |

These several observations, histological, grossly visible growth and healing, functions such as ovulation and response to hormonal stimulus lead us to assume that our preparations provide a sufficiently normal milieu to make the data obtained applicable without too many reservations to understanding of the normal processes.

[6] Extracted and assayed as reported by Werthessen, Baker and Borci in 1947.
[7] Sections of this ovary shown in Figure 5.

FIGURE 6

*Control, Human Muscle.* These sections show for the most part skeletal muscle, fibro-fatty supporting stroma, nerve tissue and blood vessels. The skeletal muscle shows appreciable edema with conspicuous outlining of the individual muscle fibers and sarcolemal surrounding sheath. The individual fibers have in the longitudinal axis the normal cross striations. The nuclei are peripherally placed, oval and only fairly well preserved. In cross section there is appreciable fragmentation of the fibers. The stroma likewise shows edema and is made up mainly of loose fibrous connective tissue. Fat is not remarkable. Nerve elements are not unusual. Blood vessels are for the most part thickwalled and have a narrow lumen. Exudative cells are not present. In an occasional field appreciable degeneration is seen so that the cells appear to have lost their normal topography and become fragmented and almost homogeneous in appearance. No tumor is identified.

*Experimental, Human Muscle.* These sections show almost complete necrosis of the tissue elements. The transformation is such that the muscle elements are replaced by agglutinated and conglutinated blood through which irregular fibrillar streaks are seen and an occasional fibroblast is identified. Small blood vessels resembling capillaries lie here and there. To one side of these sections one area showing loose fibrous connective tissue with fusiform cells and a few round cells can be made out. The last mentioned tissue appears viable but resembles an inflammatory reaction and not neoplasia.

While tissue culture techniques have achieved growth of cells and in many instances remarkable developments of structure the cited experiments are the first in our knowledge wherein growth, repair, and function of whole adult organs has been at least partially maintained over a period longer than 24 hours.

There is, therefore, now available the elements of a technique which on further development will permit the study of isolated organ systems in a close to normal milieu.


## SUMMARY

A technique for the maintenance of whole organs in a perfusion pump has been described. At its present stage of development growth of the organ and repair of injuries due to biopsies have been noted.

The extent of time during which the organ can be maintained is dependent only on the development of an uncontrollable infection.

The perfusion medium contained erythrocytes. The pump employed had a relatively large capacity permitting the circulation of the fluid at proper arterial pressure without ligation of arterioles. The high flow rate resulting prevented sedimentation of the red cells.

To the use of erythrocytes in the perfusion medium, the high flow rate and maintenance of normal arterial pressure were ascribed the ability to observe growth in the preparations.

The pump as finally developed was of simple construction and permitted easy access to the perfusion fluid and organ during the course of an experiment.



## ACKNOWLEDGMENTS

I wish to thank Mr. Myles Morgan of the Morgan Construction Company for his invention of the driving mechanism employed in this technique.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# THE RELATIONSHIP OF TEMPERATURE AND INSULIN DOSAGE TO THE RISE IN PLASMA AMINO NITROGEN IN THE EVISCERATED RAT[1]

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THAT INSULIN will depress the rate of accumulation of amino acids in the blood of eviscerated rats was reported by Frame and Russell (1946) and confirmed by Ingle, Perstrud, and Nezamis (1947). However, the results of the latter authors differed from those of Frame and Russell in two respects: The rate of increase of the blood amino nitrogen content observed by Ingle et al. was about one third of that reported by Frame and Russell, and the rise was suppressed completely by much smaller doses of insulin than those which Frame and Russell found to suppress it only partially. Ingle et al. demonstrated that these differences were not the result of their having employed complete rather than functional evisceration, nor of the use of fed instead of fasted rats, nor of hemodilution. They suggested that possibly the differences could be accounted for by differences in methods of chemical analysis, for they had used for the estimation of amino nitrogen the ninhydrin-carbon dioxide method of Hamilton and Van-Slyke, whereas Frame and Russell had employed the photometric $\beta$ naphthoquinone method, which is less specific. Another difference in the techniques used by two groups of investigators was the temperature at which the eviscerated rats were kept during the period of observation. Ingle et al, kept their animals in a chamber maintained at 26°C; under these conditions, the body temperatures of eviscerated rats fall in a short time to within a degree or two of that of the ambient air. Frame and Russell maintained the body temperatures of their animals at about 38°C.

It has now been found that the differences in the results of the two groups of experiments may be accounted for in large part, or perhaps completely, by the differences in body temperatures at which the observations were made. A small increase in the body temperature of the eviscerated rat will not only increase the glucose tolerance, as

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[1] The work reported in this paper was done under a grant from the American Cancer Society on the recommendation of the Committee on Growth, National Research Council.

Ingle noted, but will increase the rate of release of amino acids into the blood. The absolute depression of the rise in plasma amino nitrogen by a given dose of insulin at 27° and 38° is of the same order of magnitude, but since the increases without insulin are so much less at the lower temperatures, the relative response to insulin here appears to be greater than at the normal body temperatures.

## METHODS

Adult male white rats 3 to 4 months old and weighing about 350 gm. were fasted 18 hours and then functionally eviscerated under pentobarbital anesthesia. In this operation the intestinal tract, spleen, and pancreas are removed, and the liver, left *in situ*, is effectively excluded from the circulation. The operation, which takes only a few minutes, can be performed without preliminary ligation of the vena cava and without any hemorrhage either at the time of operation or later. The initial dose of sodium pentobarbital, 4 mg. per 100 gm. given intrapertioneally 10 to 20 minutes before operation, sufficed for continued light anesthesia throughout the experiment. Rectal temperatures, deep within the pelvis, were measured at frequent intervals. They were maintained at 38 ± 0.5°C by means of lights placed 30 to 40 cm. above the animals. In the series in which lower temperatures were desired, the animals were exposed at a room temperature of about 24°C; here the body temperatures fell slowly during the interval between 1 and 4 hours after evisceration from about 30° to about 28°C. The rectal temperatures were found at the end of the experiments to coincide with those elsewhere in the body.

Glucose and insulin were given to the animals subcutaneously at hourly intervals beginning 15 to 30 minutes after evisceration. Glucose solution, 4 to 18 per cent in physiological saline, was injected in two portions totaling in volume 0.5 ml. per 100 gm. per hour, the insulin (Lilly amorphous or zinc crystalline) also in saline, in a volume of 0.1 ml. per 100 gm. per hour at a separate injection site. The amount of glucose given was adjusted to the amount of insulin so that the blood sugar level was maintained within the normal range. Animals not given insulin or given not more than 0.05 units per kg. per hour received 20 mg. glucose per 100 gm. per hour; those given 0.1 unit insulin per kg. per hour required 25 to 30 mg. glucose per 100 gm. per hour, 0.25 units, 40 mg. glucose, and 1 unit insulin, 80 to 85 mg. glucose. Blood glucose determinations were made at intervals during and at the end of the periods of observation. Analyses were made on 0.1 ml. of blood by the Nelson-Somogyi method. With the regimens described above, the blood sugar concentrations were in most cases between 90 and 140 mg. per cent and appeared to remain fairly uniform throughout each experiment.

Estimations of amino nitrogen were made on plasma rather than on whole blood, inasmuch as the high content of amino nitrogen in the red cells appears not to change much except in terminal anoxia. Analyses were made on 0.2 ml. samples of oxalated plasma by the photometric procedure of Frame, Russell, and Wilhelmi (1943), as modified by Russell (1944).[2] In these experi-

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[2] For the preparation of the tungstic acid filtrates from plasma, the following procedure was used: 0.2 ml. plasma was mixed in 5.0 ml. of 0.01 N sulfuric acid, then 0.2 ml. of 3.0 per cent sodium tungstate was added and mixed immediately. After the mix-

ments, observations were made at 1 hour and again at 4 hours after evisceration, and the increases in plasma amino nitrogen occurring during the three hour interval compared in the several series of experiments.

Blood samples were taken from the tail except at the end of the experiment, when they were usually drawn from the inferior vena cava. All animals were carefully observed during the experiment and at its end, and any showing any signs of anoxia or hemorrhage, or in which the body temperatures were out of the desired range, were discarded.

Ingle et al. reported much longer survival after evisceration when the rats were kept at lower temperatures, this being the reason for their having carried out their experiments under these conditions. No attempt was made in the present experiments to determine the survival times of the animals. A distinct difference in the conditions of the animals at the higher and lower temperatures was observed, however. The animals which were kept warm remained in excellent condition throughout the experiments. Those maintained at room temperature frequently shivered, exhibited peripheral vasocontriction so that they could not be made to bleed from the cut tail, and in many cases may have been in some degree of shock. Despite the longer survival of the cold animals, it may be doubted that their state was as nearly normal as that of the rats which were kept warm.

## RESULTS AND DISCUSSION

A comparison of the rate of rise of the plasma amino nitrogen after evisceration at 38°C with that at 29°C is shown in Table 1, where it may be seen that the increase at the higher body temperature is greatly in excess of that at the lower. The temperature coefficient would be for the conventional $Q_{10}$, 2.6 fold; or more exactly, if the slope of the line relating the natural logarithm of the plasma amino nitrogen rise to the temperature is calculated, $9.1 \pm 0.7$ per cent per degree centrigrade (see Figure 2). This increase in rate with temperature is of the same order of magnitude as that commonly observed for a wide variety of chemical reactions and physiological processes and would be an expected value for the continuing net enzymatic breakdown of tissue proteins.

The increase in plasma amino nitrogen in the eviscerated rat at 38°C reported here, about 14 mg. per cent in 3 hours, agrees well with that observed previously by Frame and Russell, about 18 mg. per cent in 4 hours. Ingle, et al. found an average increase of whole blood amino nitrogen of 5.5 mg. per cent in the first 6 hours after operation and 18.5 mgm. per cent in 24 hours, at body temperatures near 27°C. These figures would correspond to increases in plasma amino nitrogen of about 4.6 and 3.9 mgm. per cent in 3 hours, over the two time intervals respectively, if the average hematocrit were taken to be 40

ture had been let stand until settling had begun, the precipitated proteins were centrifuged off, and duplicate 2 ml. portions of the supernatant were used for analysis. With the naphthoquinone amino nitrogen method, to obtain quantitative recovery of added amino acids it is essential to use less tungstate for plasma than is ordinarily used for whole blood.

per cent, and if no increase occurred in cell amino nitrogen. These figures are to be compared with a mean increase of 5.9 mgm. per cent (standard deviation 1.1 mgm. per cent) obtained in 3 hours at 29°C in the present experiments. It is clear that the rate of increase in amino nitrogen observed here at the lower temperature does not differ from the rate observed by Ingle et al. by more than can be accounted for by the difference between the expected rates at 27° and 29°. The differences in rates previously reported are not the results of differences in techniques, but are most probably solely the result of differences in the temperatures of the animals during the periods of observation.

In the previous publication the effects on the blood amino nitro-

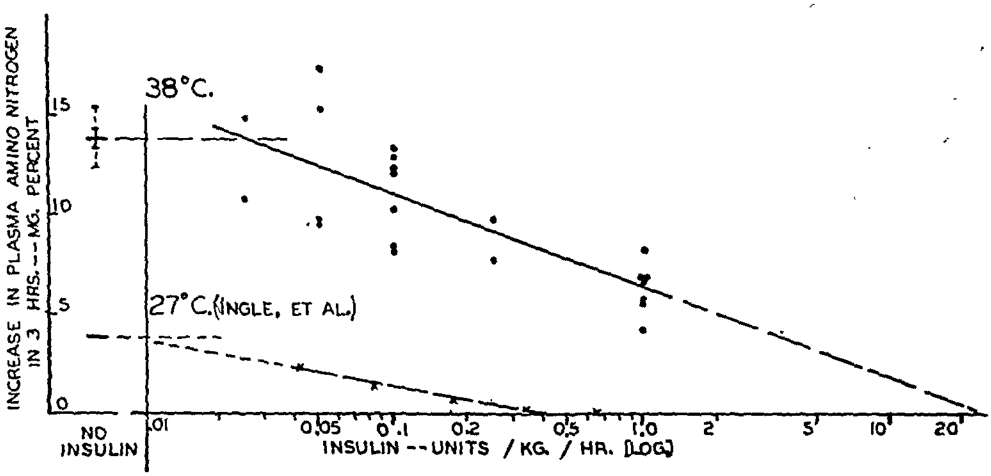


FIG. 1. The relationship of insulin dosage to the increase in plasma amino nitrogen in the eviscerated rat.

The average increase without insulin at 38° C. is given with its standard error and the standard deviation of the series. The slope of the solid line is that of the regression coefficient calculated from the individual points, each taken from a single experiment, $-4.5 \pm 0.8$. The average values indicated in the lower part of the figure were recalculated from the data of Ingle, Prestrud, and Nezamis.

gen of only one dose of insulin, 2 units per kg. per hour given intravenously to animals kept at 38°C, was described. Ingle, et al., reported the effects of a range of doses, 0.25 to 16 units per rat (250 gm.) per day, or 0.04 to 2.67 units per kg. per hour, in rats kept at 27°C. Where at the higher temperature, only partial suppression of the amino nitrogen rise was obtained with 2 units per kg., complete suppression was reported with less than 1 unit per kg. per hour at the lower temperature. In Figure 1 is presented a log.-dose response curve obtained in animals kept at 38°C, in which increases in plasma amino nitrogen are related to doses of insulin from 0.025 to 1.0 unit per kg. per hour. For comparison, data taken from the figures of Ingle, et al., from animals kept at 27°C, are also presented. The slopes of the two lines and the positions with respect to dosage are similar, so that the absolute depression of the amino nitrogen rise induced by

any given dose of insulin is of the same order at the two temperatures. To reach the same degree of repression, such as 50 per cent inhibition, however, something like 10 times as much insulin would be required at the higher temperatures, and to prevent any increase at all, on the order of 25 units per kg. per hour would appear to be required, as compared to the 0.67 units observed by Ingle, et al., to be fully effective at the lower temperature.

As shown in Table 1, in confirmation of Ingle, et al., when the animals were maintained below 29°C, 1 unit insulin per kg. per hour was found to repress nearly completely the increase in amino nitrogen which otherwise occurred. This observation indicates again no essential difference between the results of Ingle, et al., and those of Frame and Russell except those due to temperature.

From the data summarized in Table 1, curves were constructed

TABLE 1. EFFECT OF TEMPERATURE AND INSULIN ON THE INCREASE IN PLASMA AMINO NITROGEN IN EVISCERATED RATS

| | Increase in plasma amino nitrogen in 3 hours (1st to 4th hour after operation) | | | |
|---|---|---|---|---|
| | At 29°C | | At 38°C | |
| | No. obs. | mg. per cent increase | No. obs. | mg. per cent increase |
| 1. No insulin | 7 | 5.9 ± 0.41* | 11 | 13.8 ± 0.49 |
| 2. Insulin, 0.1 unit per kg. per hour (amorphous) | | | 7 | 11.0 ± 0.79 |
| 3. Insulin, 1 unit per kg. per hour | | | | |
| a. Amorphous insulin | 7 | 0.8 ± 0.43 | 7 | 6.2 ± 0.42 |
| b. Zn-crystalline insulin free of glycogenolytic factor | | | 6 | 4.5 ± 0.84 |

* Standard error.

relating the natural logarithm of the amino nitrogen increase to temperature in the presence of insulin, as well as in its absence (Figure 2). Another group of experiments not shown in Table 1 was also included; here, when the body temperatures were maintained at 33°C, the average increase in plasma amino nitrogen in 3 hours was 1.76 ± 0.53 mg. per cent. The slope of the line in the presence of insulin was 25.4 ± 6.5 per cent per degree centrigrade, significantly greater than the figure of 9 per cent seen in the absence of insulin ($t = 2.54$, with 35 degrees of freedom; $p < 0.02$). The $Q_{10}$ in the presence of insulin was approximately 7 fold. There is a suggestion here that the presence of insulin alters the reactions which limit the rate of protein breakdown. The data obtained with insulin are insufficiently exact, however, to allow any further characterization of the reactions affected.

It may be noted that although at 38°C exceedingly large amounts of insulin would be required to suppress completely the changes in blood amino nitrogen which occur after evisceration, a significant degree of depression may be obtained under these conditions with

doses of insulin which may be considered physiological. With 0.1 unit insulin per kg. per hour, the average difference of the increase in amino nitrogen from that seen in untreated animals was $-2.8 \pm 0.88$ mg. per cent,[3] or about a 20 per cent diminution in rate of release of amino acids. The decrease observed with 1 unit of insulin per kg. per hour was of the order of 55 per cent (Table 1).

While the major observations reported here were made over the

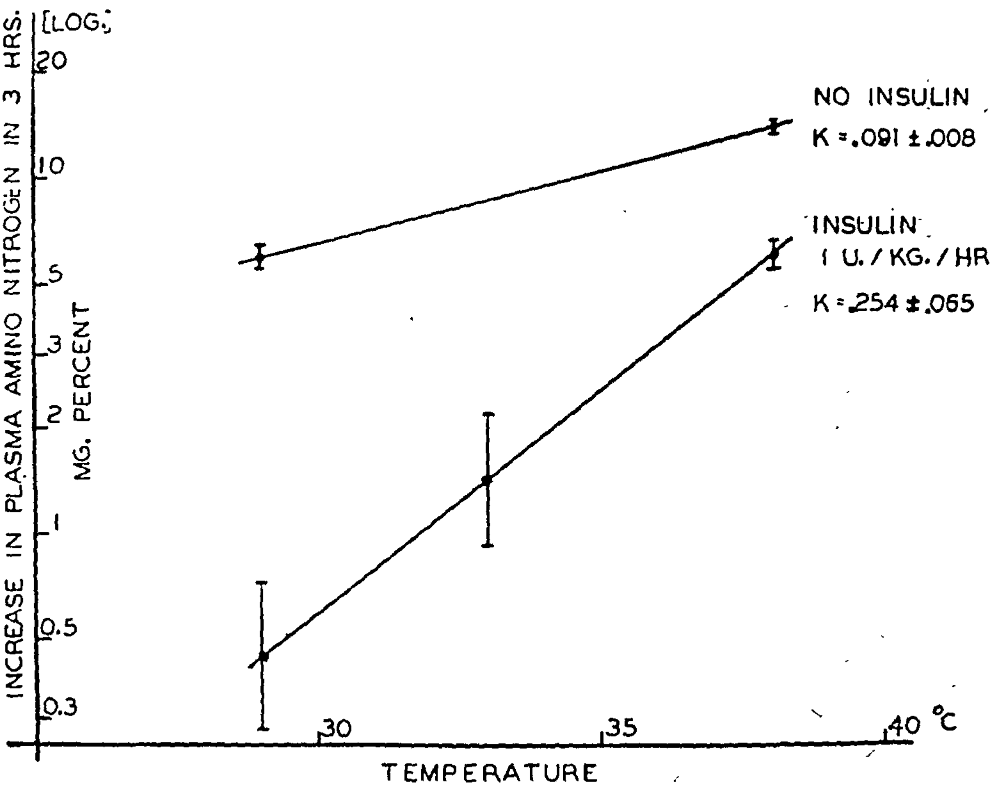


FIG. 2. The relationship of temperature on the rate of increase in plasma amino nitrogen in the eviscerated rat.

Mean values for each point, their standard errors, and the slopes of the lines (K) were calculated from the individual figures translated to logarithms. $K$, given in natural log. units, is the fractional increase in rate per °C. from the equation: $v = Ae^{Kt}$ or $\ln v = A' + K\ t$, where $v$ = velocity and $t$ = temperature. (The relationship of $K$ to the Arrhenius coefficient or "temperature characteristic," $\mu$, is: $K = \mu / R\ T^2$).

interval between 1 and 4 hours after evisceration, the effect of insulin on the plasma amino nitrogen was in fact observable at an earlier time. This may be seen in a comparison of the initial plasma amino nitrogen concentrations, determined 1 hour after evisceration and 30 to 40 minutes after the first administration of insulin. For the controls, receiving no insulin, this value was $10.0 \pm 0.4$ mg. per cent, while for the animals given 1 unit of amorphous insulin it was $6.9 \pm 0.36$ mg. per cent.

[3] $t = 3.2$, with 16 degrees of freedom; $p < .01$.

A sample of insulin stated to be virtually free of the glycogenolytic factor commonly found in insulin preparations[4] was also tested for its ability to diminish the extent of the increase in plasma amino nitrogen after evisceration. As shown in Table 1, there was no significant difference between the effects of this preparation and those of the usual amorphous insulin.

SUMMARY

The amino nitrogen content of the plasma of eviscerated rats maintained at 38°C increased an average of 14 mgm. per cent in 3 hours (between the 1st and 4th hours after operation). When the body temperatures of the rats were kept at about 29°C, the increase was reduced to 6 mgm. per cent. The calculated change in the rate of increase was then +9 per cent per °C increase in temperature. These figures indicate no essential difference between the results previously published by Frame and Russell and by Ingle, Prestrud and Nazemis, except those due to differences in the temperatures of the animals in the two series of experiments.

In eviscerated rats with body temperatures at 38°C, 1 unit of insulin per kg. per hour depressed the rate of increase in plasma amino nitrogen about 55 per cent, and 0.1 unit about 20 per cent. A log.-dose response curve relating insulin dosage to the increase in plasma nitrogen at 38°C is presented. At 29°C, 1 unit of insulin per kg. per kg. per hour prevented nearly completely the usual increase in plasma amino nitrogen. From 10 to 50 times as much insulin appear to be required to obtain the same relative effect on the plasma amino nitrogen at body temperatures of 38°C as at temperatures below 30°C.

Insulin freed of the pancreatic glycogenolytic factor was equally as effective as ordinary insulin in inhibiting the release of amino acids from the tissues.

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[4] This insulin (sample No. T2344, 26 units per mg.) was kindly furnished to Dr. Joseph Sokal by Dr. E. U. Campbell of the Lilly Company.

# THE INTERVAL BETWEEN NORMAL RELEASE OF OVULATING HORMONE AND OVULATION IN THE DOMESTIC HEN[1]

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## INTRODUCTION

THE RELEASE of ovulating hormone in the chicken (as well as in certain other spontaneously ovulating forms) may be conceived of as taking place either as an instantaneous discharge from the pituitary, or as a slow discharge over a period of one to several hours. Although we are inclined to the view that release in the chicken is more of an instantaneous than a slow discharge, we are unable to say now that it takes place definitely in one way or the other. Because of this, the term *release* in this and the accompanying paper (Rothchild and Fraps 1949) is used with certain reservations. By it is meant that point in the process of ovulation at which an amount of ovulating hormone adequate to cause ovulation is present in the blood stream, regardless of whether this amount left the pituitary immediately or over a period of some hours before.

Data on the time of release of ovulating hormone in the chicken are important, for the following reason. Enough information has been accumulated about the reproductive cycle in the chicken (Atwood, 1929; Warren and Scott, 1935; Phillips and Warren, 1937; Heywang, 1938; Warren and Scott, 1936; Byerly and Moore, 1941; McNally, 1946; Fraps, Neher, and Rothchild, 1947) to show an obvious relationship between daily cyclic changes in certain environmental factors and the characteristic rhythms of ovulation and lay. Inasmuch as there is a relationship between time of ovulation and environmental factors, the influence of the latter must undoubtedly be greater on the beginning of the process, i.e., release of ovulating hormone, than on the end of it, ovulation itself. Knowledge of the time of release, even partial knowledge, is thus helpful in the interpretation of the role that environmental factors play in the control or modification of the chicken's reproductive cycle.

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[1] This study is an enlargement of a preliminary one reported on at the 43rd annual meeting of the American Society of Zoologists (Rothchild, 1946). It is part of a study on the nature of the pituitary control over the processes of follicular maturation and ovulation in the domestic hen.

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*The main features of the ovulation and laying cycles.* (The authors cited in the preceding paragraph should be referred to for details.) The most obvious general feature of the reproductive cycle is the lay of eggs on successive days, each egg being laid at a successively later hour on each day than the one preceding it. The whole sequence of eggs is called a clutch. The initial egg of the clutch is usually laid before noon, the majority of successive intra-clutch eggs close to noon, while the terminal egg of the clutch is laid late in the afternoon. On the second day following that on which the terminal egg was laid, an egg laid comparatively early in the day initiates a new clutch. Clutches may vary in length from one to more than a dozen eggs, but in general, clutches of the same length follow one another in any particular bird.

The ovulation of each egg antedates its time of lay by an interval of roughly 24 to 28 hours. Successive ovulations ($C_s$ ovulations) within a clutch, including the terminal ovulation ($C_t$ ovulation), occur within an hour (average 30 minutes) following lay of the preceding egg. The initial ovulation ($C_1$ ovulation) of a clutch, however, always occurs at a comparatively early hour of the day (about 5:00 A.M.), about 18 hours or 18 hours plus some multiple of 24 hours following the lay of the terminal egg of the preceding clutch.

## METHODS AND MATERIALS

*Basis of the experimental procedure used.* Hypophysectomy was performed at various intervals before an expected ovulation. If the expected ovulation actually occurred following the operation, it was assumed that release of the ovulating hormone had taken place at some time prior to the operation; conversely, if it did not occur, the assumption was that the complete release of hormone, or release of an amount adequate for ovulation, was expected at some time after the time of operation. The intervals between hypophysectomy and actual or expected ovulation were then calculated, and the relationship between these intervals and the percentage of birds ovulating was used as the basis for estimating the interval between assumed time of release of hormone and ovulation. A possible alternative interpretation of the results obtained by this method is given in the discussion of the paper accompanying this one (Rothchild and Fraps, 1949).

*Determination of actual or expected ovulation time.* Time of actual ovulation was estimated from the position of the resultant egg in the oviduct at the time of autopsy. For eggs found in the magnum or isthmus of the oviduct, a curve relating proportion of the oviduct traveled to time after ovulation was used (Warren and Scott, 1935; unpublished data from this laboratory). For eggs found in the uterus, a curve relating increase in weight of the albumen to time in the uterus was used (Burmeister, 1940). For eggs found broken in the body cavity, the same procedure was used that served to estimate the time of expected ovulation in the birds that failed to ovulate.

Time of expected $C_1$ ovulation in birds that failed to ovulate was estimated by means of an average interval between ovulation and oviposition of the $C_1$ egg. This interval varies as follows in relation to

clutch length: 1 egg clutches—29 hours; 2 egg clutches—27.5 hours; clutches of 3 eggs or more—27 hours (unpublished data from this laboratory). In the series of birds used in this study, the expected time of oviposition of the egg that failed to ovulate was assumed to be the same as the time of oviposition of the last $C_1$ egg that was actually laid; the time of expected ovulation was then determined by subtraction of the appropriate ovulation-oviposition interval from this time of estimated oviposition. An example may serve to clarify this. A bird that had just laid out a 3 egg clutch was hypophysectomized at 10:00 P.M. following the lay of the last egg of the clutch, and on autopsy the next day was found to have failed to ovulate. The time of the lay of the first egg of the clutch that preceded hypophysectomy was 8:00 A.M. The time of expected lay of the first egg of the succeeding clutch, whose ovulation had been prevented by hypophysectomy, was therefore assumed to be 8:00 A.M. Since the last clutch was 3 eggs in length, 27 hours were subtracted from 8:00 A.M. giving a time of expected ovulation of 5:00 A.M.

A simpler procedure was used to estimate the time of expected ovulation of $C_s$ follicles that failed to ovulate. A $C_s$ ovulation, as mentioned above, follows a preceding oviposition by an average interval of 30 minutes. The time of expected ovulation was therefore taken as 30 minutes from either a) the actual time of lay of the oviducal egg present at the time of hypophysectomy in those birds that laid the oviducal egg at the expected normal time, or b) the time of expected normal lay of the oviducal egg in those birds that showed a delay or other alteration from the normal in the lay of this egg (as some birds do following hypophysectomy—Rothchild, 1946).

*General procedures and materials.* The birds were regularly laying adults less than 2 years old, of the Rhode Island Red and White Leghorn breeds mainly, but with a few crossbreds included. They were maintained in individual cages in laying batteries on a 14 hour light day (onset of light at 6:00 A.M. EST), and allowed *ad libitum* access to a standard laying mash and water. The laying record preceding hypophysectomy was known for each bird used, since egg collections are recorded on the hour every hour from 8:00 A.M. to 4:00 P.M. and once again at 8:00 P.M. daily, as a routine laboratory procedure.

Each bird was allowed to remain undisturbed in its cage until the actual time of operation. Hypophysectomy was performed in the majority of cases by the transbuccal method described by Hill and Parkes (1934) and in the remainder by a modification of this technique developed by one of us (Rothchild, 1948). Following operation, which in no case required more than 15 minutes, and in almost all less than 10 minutes, the birds were returned to their cages under exactly the same conditions that prevailed before operation. Autopsies were performed 4 to 16 hours after hypophysectomy, the time of autopsy for each bird being such as to permit the optimum interval to elapse after

the time of expected ovulation in those birds that had apparently failed to ovulate and a minimum interval after ovulation in those birds that ovulated.

Birds found at autopsy to have been incompletely hypophysectomized were not included in the results if ovulation had occurred, but were included if ovulation had failed.

Preliminary runs demonstrated that failure of $C_1$ ovulation was uniform when hypophysectomy was performed before 10:00 P.M., but that almost all expected $C_1$ ovulations occurred when hypophysectomy took place after 2:00 A.M. All the data on the effect of hypophysectomy on the expected $C_1$ ovulation, therefore, were gathered from birds operated on between 10:00 P.M. and 2:00 A.M. In the birds hypophysectomized before an expected $C_s$ ovulation, the information gained from the studies on the $C_1$ ovulation served as a basis for adjusting the time of operation to each bird's expected time of ovulation, in order to obtain intervals between hypophysectomy and expected ovulation that were comparable with those in the $C_1$ group.

The results are presented in terms of the interval from hypophysectomy to actual or expected ovulation. To show them as simply but as accurately as possible, the birds were divided into three general groups: those showing (1) intervals of 4 hours or less; (2) intervals of more than 4 hours but less than 6 hours; and (3) intervals of 6 hours or more. The average interval in each group was calculated for the total group, and for the ovulating and non-ovulating birds within the group. (The average intervals shown for each group of birds in Tables 1 and 2 are valid cross-sections of each group, since the ovulating and nonovulating birds in each group showed almost identical average intervals.)

## RESULTS

*Hypophysectomy before an expected $C_1$ ovulation.* The pituitaries were removed from 119 birds between 10:00 P.M. and 2:00 A.M. With the exception of 2 birds, the times of actual ovulation ranged from 3:00 A.M. to 6:45 A.M. and times of expected ovulation in the nonovulating birds from 4:00 A.M. to 9:20 A.M. The two exceptions noted apparently ovulated at 1:30 A.M. and 12:00 noon, respectively. Intervals between hypophysectomy and actual or expected ovulation ranged from 10.5 hours in the bird ovulating at noon to 2.0 hours in the bird ovulating at 1:30 A.M. Except for these two birds, the range was from 9.33 hours to 3.0 hours.

The relationship between the percentage of birds ovulating and the interval from hypophysectomy to actual or expected ovulation is shown in Table 1. These results indicate that the most frequent interval between apparent release of hormone and ovulation is between 4 and 6 hours.

*Hypophysectomy before an expected $C_s$ ovulation.* The pituitaries were removed from 41 birds between 2:45 A.M. and 6:30 A.M. Times

TABLE 1.— RELATION BETWEEN THE INTERVAL FROM HYPOPHYSECTOMY TO ACTUAL OR EXPECTED OVULATION AND THE PERCENTAGE OF BIRDS OVULATING (SEE TEXT).

| Interval: Hypophysectomy to actual or expected ovulations | | | Total number of birds | | Birds ovulating | | | |
|---|---|---|---|---|---|---|---|---|
| | Average | | | | Number | | Per cent | |
| Range | $C_1$ ovulations | $C_8$ ovulations | $C_1$ ovulations | $C_8$ ovulations | $C_1$ ovulations | $C_8$ ovulations | $C_1$ ovulations | $C_8$ ovulations |
| (hours) | (hours) | | | | | | | |
| 2.0– 4.0 | 3.3 | 3.5 | 10 | 11 | 10 | 9 | 100 | 82 |
| 4.2– 5.8 | 5.0 | 5.1 | 35 | 12 | 21 | 7 | 60 | 58 |
| 6.0–10.5 | 7.2 | 7.3 | 74 | 18 | 13 | 4 | 18 | 22 |

of actual ovulation ranged from 7:10 A.M. to 11:25 A.M. and times of expected ovulation in the non-ovulating birds from 8:30 A.M. to 12:30 P.M. The intervals between hypophysectomy and actual or expected ovulation ranged from 8.5 to 2.7 hours. The relationship between the percentage of birds ovulating and the interval between hypophysectomy and actual or expected ovulation is also shown in Table 1. There is evidently no appreciable difference between $C_1$ and $C_8$ ovulations in regard to the interval between release of hormone and ovulation.

Of the 41 birds in the $C_8$ ovulation group, 25 were expected to ovulate the terminal member of the clutch. The intervals from hypophysectomy to actual or expected *terminal* ovulations were compared with the intervals from hypophysectomy to all other actual or expected $C_8$ ovulations. The numbers of birds falling into either

TABLE 2. COMPARISON OF THE RELATIONSHIP BETWEEN THE PERCENTAGE OF BIRDS OVULATING AND THE INTERVAL FROM HYPOPHYSECTOMY TO ACTUAL OR EXPECTED *terminal* OR *intraclutch ovulation.*

| Interval: Hypophysectomy to actual or expected ovulation | | | Total number of birds | | Birds ovulating | | | |
|---|---|---|---|---|---|---|---|---|
| | Average | | | | Number | | Per cent | |
| Range | terminal ovulations | intraclutch ovulations | terminal ovulations | intraclutch ovulations | terminal ovulations | intraclutch ovulations | terminal ovulations | intraclutch ovulations |
| (hours) | (hours) | (hours) | | | | | | |
| 2.7–5.0 | 4.0 | 3.7 | 9 | 8 | 6 | 8 | 68 | 100 |
| 5.5–8.5 | 6.75 | 7.0 | 16 | 8 | 4 | 2 | 25 | 25 |

group were not very large and therefore only two ranges of intervals were used: those of 5 hours or less, and those of more than 5 hours. The results are shown in Table 2.

They indicate a possibility (no more than slight, considering the number of birds involved) that if anything, the interval from release of hormone to a terminal ovulation is slightly less than it is for all other ovulations in the clutch.

## DISCUSSION

The results presented in this paper demonstrate fairly well that the interval between release of ovulating hormone and ovulation is practically the same for all members of a clutch. The successively later hours at which ovulations occur within a clutch are thus traceable to successively later releases of ovulating hormone. Thus, since ovulations occur only within a circumscribed portion of the solar day, the releases of hormone must occur within an equally circumscribed period of time. The question of why releases of ovulating hormone occur only within certain time limits resolves itself into three interdependent questions, namely—what changes in the environment and in the animal body are associated with the stimulus that leads to the earliest release of ovulating hormone? What changes occur each day that lead to releases of the ovulating hormone at successively later hours each day? And, what changes in the environment and in the animal body are associated with the failure or prevention of a release on the day of oviposition of the terminal egg of the clutch?

These questions are raised, not because they can be answered now, but in order to define in clear terms the problem we are faced with—that of determining the nature of the relationship between the environment in which the chicken lives, and its reproductive behavior. The first steps in this direction were made by the authors cited above. The present study is an additional step forward but no means a large one. From other work (Fraps, Neher and Rothchild, 1947) taken together with this one, we now know that the *general limits* of time within which the releases of hormone occur approximate those in which the dark hours or periods of non-feeding occur, and in which the body temperature of the chicken is in general below the daily average and probably restricted to the ascending portion of the body temperature curve. But to define more exactly the temporal boundaries between earliest and latest possible time of release of ovulating hormone, it is necessary to know the extent to which the method used here measures the *actual* time of release of ovulating hormone. This point is taken up briefly in the discussion of the paper accompanying this one, (Rothchild and Fraps, 1949).

## SUMMARY

Mature, regularly laying domestic hens were hypophysectomized at intervals ranging from 10 hours to 2 hours before an expected ovulation. The expected ovulation in 119 cases was that of an initial member of a clutch, and in 41 cases was that of an intraclutch or terminal member. It was found that the percentage of birds ovulating the expected ovum increased with the decrease in the interval from hypophysectomy to actual or expected ovulation, and that the most frequent interval between apparent release and ovulation was of the order of 4 to 6 hours, and was not related to position in the clutch.

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# THE INDUCTION OF OVULATING HORMONE RELEASE FROM THE PITUITARY OF THE DOMESTIC HEN BY MEANS OF PROGESTERONE[1]

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## INTRODUCTION

THE INDUCTION of ovulation in the hen by means of progesterone was first noted by Fraps and Dury (1943). Later, unpublished experiments of Fraps and his coworkers indicated that the probable cite of action of this hormone was the bird's own pituitary. These experiments showed (1) that the ovarian follicles of hens that had been treated with pregnant mare's serum could not be ovulated with progesterone, but were capable of being ovulated with luteinizing pituitary substances; and (2) that the pituitaries of these hens treated with pregnant mare's serum were considerably lower in ovulating potency than the pituitaries of normal laying hens.

The present studies were undertaken to determine as conclusively as possible whether or not progesterone induced a release[3] of ovulating hormone from the pituitary of the treated hen, and to what extent this release resembled the natural one. Because of the well known ovulation-inhibiting effect of progesterone in mammals, the question of progesterone inhibition of ovulation in chickens was also considered briefly.

## METHODS AND MATERIALS

The ovulation and egg-laying pattern of the domestic hen is described in sufficient detail in the first paper in this series (Rothchild and Fraps, 1949), for an understanding of the plan of these experiments. The test ovarian follicle in all the birds used in this study was the $C_1$ or first follicle of an oncoming clutch. This follicle ovulates normally at about 5:00 A.M. under the conditions in this laboratory, but injections of progesterone at any time after about 1:00 P.M. on the day preceding its expected ovulation induce it to

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[1] A preliminary report of these studies was made at the 31st annual meeting of the American Physiological Society (Rothchild and Fraps, 1947).

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[3] See introduction to first paper in this series (Rothchild and Fraps, 1949) for limitations on the use of this term.

ovulate at an average interval of 9.0 hours from the time of injection (see below). The normal ovulation of this follicle can be prevented uniformly by hypophysectomy carried out at 10:00 P.M. (Rothchild and Fraps 1949) or earlier on the day preceding its expected ovulation. In the present studies, except where otherwise noted, all injections took place between 1:00 P.M. and 7:30 P.M. and all operations (complete or sham hypophysectomies) between 2:20 P.M. and 10:00 P.M. on the day preceding the expected $C_1$ ovulation.

Progesterone was dissolved in propylene glycol at a concentration of 10.0 mg./ml. for intravenous use, and in corn oil at a concentration of either 10.0 mg./ml. or 12.5 mg./ml. for subcutaneous use.

The pituitary material used was a preparation of male chicken anterior pituitaries, frozen immediately after collection and dried on the same day by pressing them out on glass plates, and exposing the plates to a current of warm air. The dried powdered material was stored in a desiccator over $CaCl_2$. For use it was taken up in distilled water and injected intravenously in a volume of 0.10 to 0.20 ml. per bird (0.5 mg./ml. or 1.0 mg./ml.).

The operative technique used was hypophysectomy. It was performed in most cases by the transbuccal method described in detail by Hill and Parkes (1934). A modification of this technique developed by one of us (Rothchild, 1948) was used on a small number of birds added after the main features of the study had been carried out with the older method. Sham hypophysectomy consisted of every step of the transbuccal operation except the removal of the pituitary.

Autopsies, except where otherwise noted, took place within 10 to 18 hours from the time of hypophysectomy. The occurrence and time of actual ovulation were determined as described before (Rothchild and Fraps, 1949), the Burmeister (1940) curve[4] being used in the majority of cases. Time of expected ovulation in the non-ovulating birds and in the ovulating birds in which the yolk was found broken in the body cavity was estimated to be 9.0 hours from the time of injection of progesterone, since this was the average interval (range: 6.0–11.3 hours; most frequent interval: 8.0–10.0 hours) between injection and ovulation[4].

Incompletely hypophysectomized birds were excluded from the results if ovulation occurred, but were included if ovulation had failed.

Breeds, ages and conditions of maintenance were the same as described in the first paper of this series (Rothchild and Fraps, 1949).

## EXPERIMENTS AND RESULTS

Experiments 1, 2 and 3 deal with the mechanism of the ovulation-inducing action of progesterone in the domestic hen; experiment 4 with the ovulation-inhibiting action of progesterone.

*Experiment 1* was set up to determine whether progesterone would induce ovulation in the absence of the pituitary. The plan and results

[4] Use of the Burmeister curve would presumably involve a greater error in determination of ovulation time than the use of the method involving the location of the egg in the magnum. However, in 12 control birds, ovulation time as determined by the latter method averaged 8.33 hours from time of injection of progesterone—a value which is certainly of the same order as that determined by means of the Burmeister curve.

of the experiment are presented in Table 1. These demonstrated very clearly that the absence of the pituitary completely abolished the ovulation-inducing action of progesterone.

TABLE 1. FAILURE OF PROGESTERONE TO INDUCE OVULATION IN THE HYPOPHYSECTOMIZED HEN. THE OVULATIONS NOTED OCCURRED ROUGHLY 9 HOURS AFTER THE INJECTION OF PROGESTERONE, OR ABOUT 6 HOURS BEFORE THE EXPECTED NORMAL TIME.

| Progesterone dose per bird | Route of administration | Time of hypophysectomy | Birds treated | Birds ovulating | |
|---|---|---|---|---|---|
| (mg.) | | | (no.) | (no.) | (%) |
| 1.0 | intravenous | unoperated on | 30 | 25 | 83 |
| 10.0 | intravenous | unoperated on | 7 | 7 | 100 |
| 10.0 | intravenous | immediately before injection | 10 | 0 | 0 |
| 10.0 | intravenous | 2 hours after injection | 11 | 0 | 0 |
| 10.0 | subcutaneous[1] | 2 hours after injection | 5 | 0 | 0 |
| 1.0 | intravenous | *sham hypophysectomy* immediately before injection | 11 | 11 | 100 |

[1] No controls were run on this level of progesterone by this route of administration but in Fraps and Dury's paper (1943) between 90%–100% of birds ovulated a $C_1$ follicle in response to subcutaneous injection of 1.0–10.0 mg. of progesterone.

*Experiment 2* was set up to determine whether the ovarian follicle was capable of ovulating in the absence of the pituitary. The experimental plan and results are shown in Table 2. Although a decrease in responsiveness of the follicle occurred immediately after hypophysectomy, it was still able to ovulate in response to sufficiently large doses of pituitary powder, a result quite different from that obtained with progesterone.

TABLE 2. ABILITY OF THE OVARIAN FOLLICLE TO OVULATE FOLLOWING HYPOPHYSECTOMY.

| Dose of pituitary[1] per bird[2] | Time of hypophysectomy | Birds treated | Birds ovulating | |
|---|---|---|---|---|
| (mg.) | | (no.) | (no.) | (%) |
| 0.05 | unoperated on | 35 | 28 | 80 |
| 0.05 | immediately before injection | 19 | 2 | 10 |
| 0.10 | immediately before injection | 21 | 11 | 52 |
| 0.20 | immediately before injection | 10 | 8 | 80 |

[1] See Methods and Materials.
[2] All injections were made intravenously in this series of birds.

The two experiments taken together indicated that the failure of ovulation in the hypophysectomized bird following progesterone injection was most probably due to a lack of pituitary action on the follicle and, therefore, that progesterone in all likelihood induced ovulation by stimulating a release of ovulating hormone from the pituitary.

*Experiment 3* was carried out (a) to determine the time required

for the completion of the assumed release of ovulating hormone following progesterone injection, and (b) to compare (1) the interval from time of assumed release induced with progesterone to ovulation with (2) the interval from the time of natural release to ovulation.

Progesterone was injected intravenously, the pituitaries removed up to and including 4.0 hours afterwards, and the incidence and time of ovulation noted. In Table 3 the incidence of ovulation in relation to the interval from injection to hypophysectomy is summarized; evidently the pituitary must be left in situ for 4 hours following the injection of progesterone for a maximum normal ovulating response to take place. The significance of this is taken up in the discussion.

TABLE 3. RELATION BETWEEN THE INTERVAL FROM PROGESTERONE INJECTION (INTRAVENOUS) TO HYPOPHYSECTOMY AND THE INCIDENCE OF OVULATIONS

| Time from progesterone injection to hypophysectomy | Amount progesterone injected | Birds treated | Birds ovulating | |
|---|---|---|---|---|
| (hours) | (mg.) | (no.) | (no.) | (%) |
| 0—less than 2[1] | 1.0 | 16 | 0 | 0 |
| | 10.0 | 10 | 0 | |
| 2 | 1.0 | 11 | 2 | 9 |
| | 10.0 | 11 | 0 | |
| More than 2 to less than 3[2] | 1.0 | 21 | 8 | 38 |
| 3 | 1.0 | 15 | 10 | 67 |
| 4 | 1.0 | 17 | 14 | 82 |

[1] Hypophysectomy immediately before progesterone injection to 101 minutes after.
[2] Hypophysectomy between 132 and 166 minutes after progesterone injection (average—150 minutes).

Table 4 shows the relationship between the interval from hypophysectomy to expected or actual ovulation and the percentage of birds ovulating in response to progesterone, in comparison with the same relationship in birds ovulating under natural conditions. The time relations involved are practically identical for both naturally-induced

TABLE 4. NATURALLY-OCCURRING AND PROGESTERONE-INDUCED OVULATIONS COMPARED FOR THE RELATIONSHIP BETWEEN THE PERCENTAGE OF BIRDS OVULATING AND THE INTERVAL FROM HYPOPHYSECTOMY TO ACTUAL OR EXPECTED OVULATION.

| Interval: Hypophysectomy to actual/expected ovulation | | | Total number of birds | | Birds ovulating | | | |
|---|---|---|---|---|---|---|---|---|
| Range | Average | | | | Number | | Percent | |
| | Natural[1] | Progesterone[2] | Natural[1] | Progesterone[2] | Natural[1] | Progesterone[2] | Natural[1] | Progesterone[2] |
| (hours) | (hours) | (hours) | | | | | | |
| 2.0– 4.0 | 3.5 | 3.3 | 21 | 5 | 19 | 5 | 90 | 100 |
| 4.2– 5.9 | 5.1 | 5.3 | 47 | 21 | 28 | 15 | 60 | 71 |
| 6.0–10.5 | 7.2 | 7.3 | 92 | 77 | 17 | 16 | 18 | 21 |

[1] Ovulations occurring under natural conditions, i.e., normally expected $C_1$ and $C_s$ ovulations together (see Rothchild and Fraps, 1949).
[2] Ovulations induced with progesterone.

and progesterone-induced assumed releases of ovulating hormone.

*Experiment 4*, as indicated above, involves the inhibition of ovulation with progesterone. It has been known for some time in this laboratory that although progesterone will induce ovulation, at least the oldest follicle in some birds that fail to ovulate, and some or all of the younger follicles in those birds in which ovulation occurs, will undergo atresia. This effect takes place even with dosages of progesterone as low as a fraction of a milligram. The action of progesterone on follicles *less mature* than the ovulable one is thus evidently primarily an ovulation-inhibiting one.

To demonstrate this even more conclusively, 15 birds were selected for particularly regular lay records, and at 4:00 P.M. on the day of ovulation of a terminal follicle, i.e., roughly 37 hours before the next expected ovulation, subcutaneous injections of progesterone were begun. A total dose of 12.5 mg. of progesterone per bird per day was given, divided into two injections, at 4:00 P.M. and 8:00 A.M. In 10 birds the injections were continued until 4:00 P.M. of the day of expected $C_1$ ovulation, and the birds were killed at 8:00 A.M. the next day, roughly 28 hours after the expected ovulation. The remaining 5 birds were killed exactly 24 hours earlier than this, roughly 3 hours after the time of expected ovulation. In all 15 birds ovulation failed to occur. In 5 of the 10 birds killed 28 hours after expected ovulation, and in 3 of the 5 birds killed 3 hours after expected ovulation, the follicles were in atresia.

Although this experiment is admittedly crude, it demonstrates the difference in the effect of progesterone when injected at different intervals before an expected ovulation. The same *level* of progesterone, or one slightly less than this (10.0 mg.) when injected by either the subcutaneous or intravenous route on the day *after* the ovulation of the terminal follicle has primarily an ovulation-inducing effect.

In another connection, this same relationship was demonstrated in the laboratory recently by Mr. B. H. Neher. He injected a total of either 2.0, 4.0 or 6.0 mg. of progesterone (using 1, 2 or 3 injections of 2.0 mg. each) into 3 groups of birds. In 16 birds a single injection was made at 4:00 P.M. on the day of ovulation of the terminal egg; in 12 of these birds, a second injection was made at 12:00 midnight following the first injection; and in 8 of these birds a third injection was made at 9:00 A.M. the following day. No autopsies were carried out, but the lay records of these birds indicated an indefinite delay in ovulation or atresia (more likely the latter) of the $C_1$ follicle in 3 of the 4 birds given the single, 3 of the 4 birds given the double, and 7 of the 8 birds given the triple injection. It will be noted that the first injection in every case took place at 4:00 P.M. following the terminal ovulation. However, in another group of birds, he made a single injection at 4:00 A.M. following the terminal ovulation and in these birds the effect was not inhibition of ovulation but an induction of ovulation.

## DISCUSSION

The facts concerning the ovulation-inducing action of progesterone are these: ovulation cannot be induced with progesterone in birds whose pituitaries are low in ovulating potency (as shown by the pregnant mare's serum experiments of Fraps, et al. cited above) or in hypophysectomized birds, but progesterone does induce ovulation in normal or sham hypophysectomized birds; ovulation can be induced in birds whose pituitaries are either lacking or low in potency, by the injection of pituitary substance; the percentage of birds ovulating following progesterone injection is zero when the pituitary is removed within less than 2 hours after injection, but rises to the same value shown by normal birds when the pituitary is removed at 4.0 hours after the injection; the percentage of birds ovulating in relation to the interval from hypophysectomy to expected ovulation is practically the same for birds ovulating under natural conditions or in response to progesterone. These facts, we feel, justify the assumption that progesterone induces a release of ovulating hormone from the pituitary.

A comparison of the time relations between hypophysectomy and naturally induced ovulations, and hypophysectomy and progesterone-induced ovulations, forces on our consideration the question of whether or not the assumptions made in carrying out the experiments reported in the preceding paper were correct. These were: if hypophysectomy was followed by ovulation, the release occurred before the time of hypophysectomy; if hypophysectomy prevented ovulation the release was expected after the time of hypophysectomy. Yet the data reported in the present paper make it fairly clear (1) that progesterone stimulates a release of hormone from the pituitary, and (2) that hypophysectomy at any time within less than 4 hours following the injection of progesterone partially or completely prevents the expected ovulation. It seems rather unreasonable to assume, as an explanation of this, that the release of hormone occurs as late as 4 hours after the injection of progesterone, especially since intravenously injected progesterone probably has only a very short stay in the blood stream. What is perhaps a more likely explanation is the following. After a release of ovulating hormone, the continued secretion of a follicle-maintaining factor by the pituitary is necessary for the follicle to initiate its reaction to the ovulating hormone. The time before expected ovulation when this continued secretion becomes dispensable, then, would be the time at which hypophysectomy does not prevent ovulation, and it would follow the time of release of hormone by some amount up to 4 hours. The other side of the same coin would be, of course, that failure of ovulation following hypophysectomy could signify not only the possibility that hypophysectomy preceded the time of expected release, but also that it followed the actual release of hormone at some interval less than that required for complete mainte-

nance of the follicle in a state of reactivity to the ovulating hormone. (Some experiments in progress now indicate this as a worthwhile possibility, but since they are by no means conclusive as yet, the discussion of their significance must be left for a future date.)

As has already been mentioned (Fraps, Neher and Rothchild, 1947; Rothchild and Fraps, 1949) the release of the ovulating hormone under natural conditions takes place only within a circumscribed portion of each 24 hours. Since $C_1$ ovulation can be induced by injections of progesterone at times when naturally-induced releases of ovulating hormone do not occur, the pituitary is evidently responsive to a release stimulus at these times, and the failure of the natural release to occur may be due to a drop in or absence of activity of the normal release-provoking system at these hours of the day. It is also possible that the pituitary itself shows a cycle of responsiveness to the release stimulus, and that the failure of release at certain hours of the day is due to the fact that the pituitary has lost its responsiveness to a release-provoking stimulus. Since the action of progesterone so closely parallels that of the natural release stimulus, if it is not actually the stimulus itself, it may serve as a useful tool with which to demonstrate the existence and characteristics of such a cycle of responsiveness in the pituitary.

The belief that, in regard to ovulation, progesterone is primarily an ovulation-inhibiting hormone rests largely on the following experimental evidence. Removal of corpora lutea in the guinea pig is followed by a shortening of the estrus cycle (Loeb, 1911); the drop in ovulating potency of the estrus rabbit's pituitary that occurs following coitus does not take place following coitus of the pseudopregnant rabbit, nor does the pseudopregnant rabbit ovulate following coitus (Makepeace, Weinstein and Friedman, 1938); continued injections of crude extracts of corpora lutea or of progesterone interrupt the estrus cycles of rats and mice (Papanicalaou, 1926; Parkes and Bellerby, 1927; Hisaw, Meyer and Weichert, 1928; Haterius and Pfiffner, 1929; Patel, 1930; Selye, Browne and Collip, 1936; Phillips, 1937), prevent the induction of pseudopregnancy in rats following cervical stimulation (Astwood and Fevold, 1939), the preovulatory swelling of the Graafian follicles in the guinea pig (Dempsey, 1937) the ovulation that normally follows coitus (Makepeace, Weinstein and Friedman, 1937), or copper injections (Friedman, 1941) in the rabbit, and the luteinization of the ovary that usually follows injections of follicle stimulating hormone in the immature rat (Astwood and Fevold, 1939).

The injections of progesterone or progestin in most of these cited experiments were made in relatively large amounts, and over long periods of time. In the only experiment described in the literature in which progesterone was given in a single injection in a relatively high dose to a normal mammal, the effect of the progesterone was appar-

ently to induce an ovulation (Everett, 1944). The same author had previously shown that progesterone facilitated if it did not actually induce ovulations in constant estrus rats (Everett, 1940).[5]

In the chicken progesterone has either an ovulation-inhibiting or an ovulation-inducing effect depending on the time of administration. Practically all the evidence for progesterone's ovulation-inhibiting activity in the mammal is of the same order as that which demonstrated inhibition in the bird, i.e., injections were started relatively early in regard to time of expected ovulation, and continued for a relatively long period of time. These facts lead us to believe that the role of progesterone in the mammalian estrus cycle deserves reexamination.[5] The fact that Dempsey, Hertz, and Young (1936) found evidence for the secretion of progesterone in the guinea pig several hours before ovulation also contributes to the possibility that progesterone, in the follicular phase of the mammalian estrus cycle, may actually be involved in the induction of ovulation, while in the luteal phase one of its principal roles is that of inhibition of ovulation.

SUMMARY

An amount of progesterone 10 times that required to induce ovulation in intact normal birds was found to be completely ineffective when injected immediately after hypophysectomy; the dose required for normal birds, however, was completely effective when injected immediately after sham hypophysectomy. Removal of the pituitary within 2 hours after the injection of progesterone prevented ovulation from taking place. Hypophysectomy between 2 and 4 hours after injection permitted increasing percentages of birds to ovulate, the same percentage as normal ovulating when hypophysectomy was delayed until 4 hours after the injection. The maximum interval from hypophysectomy to actual or expected ovulation at which the maximum percentage of birds ovulated was of the order of 4 to 6 hours, which was the same value found for birds ovulating in response to a natural release of ovulating hormone. These facts justify the conclusion that progesterone induces a release of ovulating hormone from the pituitary of the chicken.

The ovulation-inhibiting effect of progesterone described for mammals was demonstrated in the chicken as well. Inhibition of ovulation in the latter follows progesterone injections made at intervals of 36 hours or greater before an expected ovulation.

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# THE INFLUENCE OF GROWTH HORMONE ON FASTING METABOLISM[1]

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THE ROLE of the adrenal cortex and of the thyroid in regulating the rate of mobilization of nitrogen from the tissues of the fasting mouse has been investigated recently by White and Dougherty (1947). It was found that the adrenal cortical steroids affected the rate of loss of nitrogen from lymphoid tissue. Mobilization of nitrogen from the carcass of the fasting mouse was negligible in the absence of the thyroid, although nitrogen loss from the liver of either thyroidectomized or adrenalectomized animals remained relatively independent of these hormonal influences. The data further suggested that translocation of nitrogen from carcass to lymphoid tissue of the fasting animal was influenced by thyroid secretion.

These attempts at localization of endocrine control over the contribution of nitrogen from specific tissues in the fasting animal are extended in the present study to the investigation of another potent endocrine influence, namely, purified growth hormone. This has been done with a view to obtaining evidence regarding the locus of the nitrogen-sparing action of this hormone. Accordingly, the effect of purified adenohypophyseal growth hormone on the net changes of nitrogen, as well as water and lipid, in specific tissues was investigated in both intact and adrenalectomized mice during a 48-hour fast. It was found that this growth-promoting preparation decreased the rate of protein utilization and accelerated lipid metabolism of control and adrenalectomized mice.

In spite of its pronounced effects on liver and carcass composition and weight loss, purified growth hormone was without influence on the metabolism of lymphoid tissue of intact mice during fasting, except at very high dose levels where augmented fasting involution of lymphoid structures was observed. Administration of growth hormone to fasting, adrenalectomized mice failed to potentiate the growth

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of lymphoid tissue. These observations would appear to lend additional support to the specificity of pituitary-adrenal cortical control over lymphoid tissue weight, structure and function.

## METHODS

Male mice of the CBA strain (Strong), between 8 to 10 weeks of age and weighing 18 to 22 grams, were employed. Preceding the fast, all animals were maintained on Purina laboratory chow pellets supplemented with powdered calf meal. The mice were kept in individual metabolism cages during fasting, with free access to water; the adrenalectomized animals received 0.9% sodium chloride as drinking fluid. Subcutaneous injection of 0.25 mgm. desoxycorticosterone acetate (in 0.05 ml. sesame oil, Schering) was used as an immediate postoperative supportive measure. Adrenalectomized mice were allowed food for 24 to 48 hours before institution of the fast.

Growth hormone[3] in physiological saline was injected intraperitoneally in a total dose of either 200 micrograms or 4 milligrams, respectively. The hormone was administered in 4 divided doses over the 48-hour fasting period. Control fasted animals were injected with saline.

### *Sampling and analytical procedures*

At the end of the fasting period, the mice were sacrificed by severing the cervical vertebrae, and samples of tissue from individual animals were obtained immediately for analysis.

a. *Lymphoid tissue*

A representative and uniform sample of lymphoid tissue, consisting of paired axillary and inguinal nodes, six cervical nodes, the mesenteric node chain, thymus, and spleen, was rapidly dissected free of surrounding fat and connective tissue. Suitable precautions were taken to prevent tissue dehydration. The lymphoid tissue was weighed immediately in a small tared vial on the analytical balance, dried at 105°C. overnight, and a dry weight determined.

In the initial studies, the water-free samples were transferred quantitatively with the aid of ethanol to a semi-micro Soxhlet appratus. Total lipid

[3] The growth hormone preparation employed was made available through the courtesy of Drs. A. E. Wilhelmi, J. B. Fishman and J. A. Russell of the Department of Physiological Chemistry, Yale University. It was derived from beef anterior pituitary tissue and had been purified by repetition of the calcium hydroxide—ethanol fractionation procedure described by Fishman, Wilhelmi, and Russell (1947). When assayed by these investigators in 100 gm. hypophysectomized rats, 10 micrograms of this preparation administered daily for 10 days permitted an average total weight gain of approximately 9 gm. The increase in width of the epiphyseal cartilage in similar animals averaged 78 micra. Comparable material assayed at the Armour Laboratories in Chicago, through the courtesy of the late Dr. Fred C. Koch, showed no demonstrable lactogenic, gonadotrophic, or posterior lobe hormonal activity. The preparation contained less than 1 per cent of the adrenotrophic activity of a standard preparation of adrenotrophin, and 0.1 Evans chick unit of thyrotrophic potency per milligram. These hormonal contaminants did not appear to be significant factors in the experiments described in the present report. This may be deduced from the high activity of the preparation in the adrenalectomized animals, and from data obtained from histological examination of thyroids of fasted, growth hormone-treated mice. The latter glands were indistinguishable from those of control fasted animals in their resting, basal appearance.

was estimated gravimetrically by a modification of the procedure described by Roberts and Samuels (1946). This included exhaustive extraction of the sample with alcohol and ether successively, and resolution with petroleum ether. The dry, fat-free tissues were transferred quantitatively to a Kjeldahl flask and digested with 2 ml. of concentrated sulfuric acid in the presence of a Hengar granule as catalyst. Digestion was completed with 5 drops of Superoxol. Suitable aliquots were analyzed for nitrogen by the method of Sobel et. al. (1937), with the exception that mixed methyl red—bromcresol green was used as indicator. The nitrogen content of the petroleum ether-insoluble fraction of the total lipid extract was also determined (lipoprotein nitrogen), and added to the above value. Total nitrogen multiplied by the factor 6.25 was used as an approximation of the protein present.

The lymphoid tissue water, nitrogen and lipid concentrations under specific conditions were strikingly reproducible. These three values accounted for 100% of the tissue composition, with a maximum deviation of 1.5%. Therefore, in later experiments it was considered unnecessary to determine total lymphoid tissue lipid; this value was derived by difference, using the observed concentrations of water and nitrogen.

b. *Liver.*

At autopsy, a sample of liver was blotted free of blood and taken for water analysis, as described above. Duplicate fresh samples, weighed rapidly on the Roller-Smith torison balance, were obtained for total nitrogen determination. The remaining liver tissue was weighed and preserved in 95% ethanol for subsequent lipid analysis as described above; these samples were ground with reagent grade sea sand prior to lipid extraction, In some cases, samples of liver were placed in 10% formalin for subsequent histological examination.

c. *Carcass.*

Following dissection of the lymphoid tissue and liver, the gastrointestinal tract was removed, weighed and discarded. The remaining carcass was frozen rapidly in a mixture of diethyl ether and solid carbon dioxide. It was then ground rapidly to a fine uniform powder in a Quaker City laboratory power mill which had been cooled previously by grinding dry ice. Prior to grinding of the carcass, excess carbon dioxide was blown out of the mill with a stream of compressed air.

Weighed samples of ground carcass were then taken for water, nitrogen, and lipid analysis by methods described above. The aliquot for total lipid was stored in 95% ethanol, and subsequently transferred directly, without further grinding, to the Soxhlet apparatus.

## RESULTS

1. *The effect of adrenalectomy on tissue composition of the fed animal.*

Intact and adrenalectomized mice were sacrificed in the fed state to provide data from which was inferred the tissue composition at the institution of the fast. Comparisons of the analytical data were made with regard to the appropriate controls: intact fasted with intact fed, and adrenalectomized fasted with adrenalectomized fed, animals.

The reason for the distinction in controls will be clear from Table 1

TABLE 1. CHANGE IN TISSUE COMPOSITION OF MALE CBA MICE 24–48 HOURS AFTER ADRENALECTOMY†

| | Intact | | Adrenalectomized | | |
|---|---|---|---|---|---|
| | Total present gm. | gm./100 gm.B.W. | Total present gm. | gm./100 gm.B.W. | % Change |
| Liver | | | | | |
| Protein | 0.296 | 1.32 | 0.252 | 1.22 | − 7.6 |
| Water | 0.955 | 4.26 | 0.792 | 3.84 | − 9.8 |
| Lipid | 0.103 | 0.46 | 0.083 | 0.40 | −13.0 |
| Carcass | | | | | |
| Protein | 4.41 | 19.7 | 4.08 | 19.8 | + 0.5 |
| Water | 14.90 | 66.5 | 13.90 | 67.5 | + 1.5 |
| Lymphoid Tissue | | | | | |
| Protein | 0.032 | 0.143 | 0.040 | 0.194 | +35.7 |
| Water | 0.152 | 0.680 | 0.191 | 0.926 | +36.2 |
| Lipid | 0.015[1] | 0.067 | 0.011[2] | 0.053 | −20.9 |

[1] By analysis.
[2] By difference, using $H_2O$ and N percentages observed.
† Numbers of animals used indicated in legend for Figure 1.

in which are tabulated the effects of adrenalectomy on the composition of lymphoid tissue, liver, and carcass of fed animals. It will be observed that loss of protein, water and lipid from the liver occurred in the adrenalectomized group. Total liver weight was thus significantly decreased in these animals with respect to the fed controls (see also Table 2). Carcass exhibited virtually no change.

TABLE 2. THE INFLUENCE OF GROWTH HORMONE ON LIVER WEIGHT IN MALE CBA MICE DURING FASTING†

| Group | No. of Animals | Total Liver Wet wt. (gm/100 gm. B.W.) | Total Liver Wet wt. (gm.) | Total Liver wt.-Fat-free (gm.) | Total Liver wt.-dry, fat-free (gm.) |
|---|---|---|---|---|---|
| *Intact* | | | | | |
| Fed | 8 | 6.07 ± 0.19 | 1.3621 ± 0.049 | 1.2592 ± 0.044 | 0.3025 ± 0.022 |
| Fasted 48 hrs. | 13 | 5.16 ± 0.16 | 0.8640 ± 0.038 | 0.7870 ± 0.030 | 0.2013 ± 0.010 |
| Fasted 48 hours.+ Growth Hormone, 200 μgm. | 14 | 6.36 ± 0.07* | 1.1110 ± 0.044* | 0.9240 ± 0.028* | 0.2278 0.010 |
| Fasted 48 hrs.+ Growth Hormone, 4 mgm. | 6 | 6.92 ± 0.31* | 1.2433 ± 0.070* | 0.9481 ± 0.028* | 0.2345 ± 0.014* |
| *Adrenalectomized* | | | | | |
| Fed | 13 | 5.43 ± 0.11 | 1.1210 ± 0.039 | 1.0380 ± 0.038 | 0.2460 ± 0.008 |
| Fasted 48 hrs. (+saline) | 11 | 4.57 ± 0.10 | 0.8896 ± 0.025 | 0.8045 ± 0.025 | 0.1732 ± 0.006 |
| Fasted 48 hrs.+ Growth Hormone, 200 μgm. | 10 | 5.28 ± 0.12* | 1.0300 ± 0.025* | 0.8710 ± 0.026* | 0.1910 ± 0.007* |

† Deviations shown are standard errors.
* The starred values are significantly different from the respective fasted control figures.

The well-known hypertrophy of lymphoid structures following adrenalectomy was also observed in the present investigation (Table 1; see also Table 5). The composition of this growth appeared to be an increase in the amounts of water and protein present, accompanied by a reduction in total lipid (Table 1). The latter, however, may be of questionable significance, because the absolute amounts of lipid estimated were very small.

These data are illustrated graphically in Figure 1.

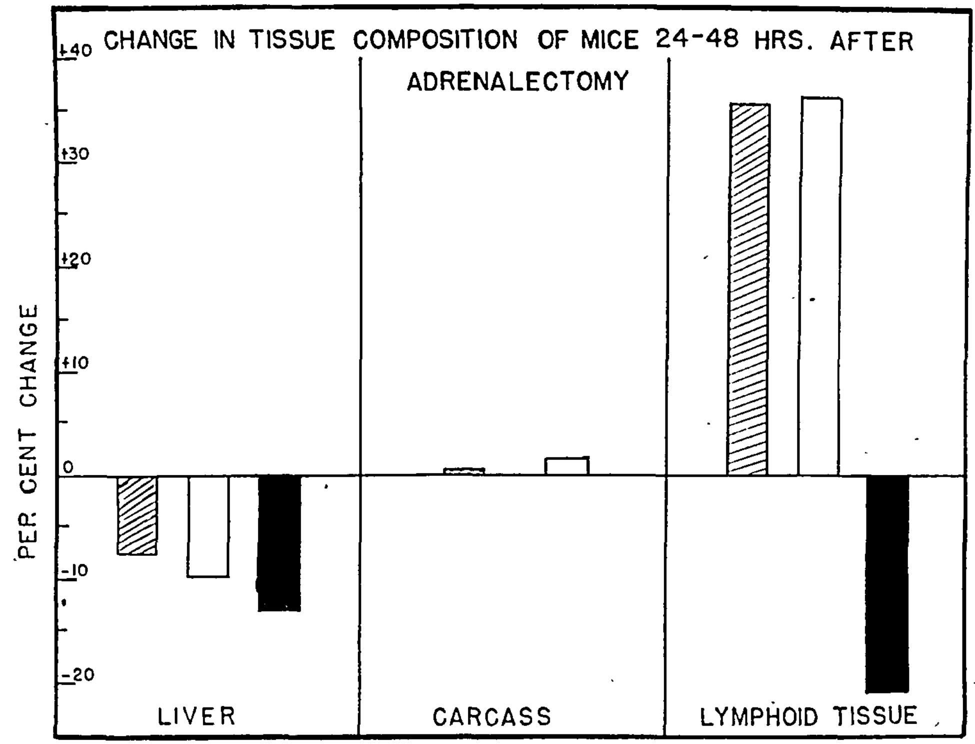


FIG. 1. The length of the bars represents the per cent change of tissue constituents in fed adrenalectomized animals, 24–48 hours after operation, related to the tissue composition of intact controls taken as a zero baseline. The cross-hatched bars represent change in protein, the open bars, change in water, and the solid bars, change in lipid. The number of individual animals contributing to the average values were 8 intact controls, 9 adrenalectomized animals used for lymphoid tissue and carcass analysis, and 13 similar animals for liver data.

2. *The influence of growth hormone on liver of fasting mice.*

a. *Weight and composition*

Intact mice fasted for 48 hours lost approximately 32% of liver protein when compared with fed controls (Figure 2). The degree of water loss was of a similar order of magnitude. If, however, growth hormone was administered during the fast, there was a statistically significant reduction in the degree of nitrogen and water loss from the liver, i.e., a relative retention of nitrogen, in the complete absence of exogenous sources of protein. This is seen Figure 2 for two doses of growth hormone in intact animals, and for the 200 microgram dose in

the adrenalectomized group. It will be noted that protein loss from the liver of adrenalectomized animals during fasting was very similar in magnitude to that observed in intact mice, as had been reported previously (cf. White and Dougherty, 1947). The ratio of water: protein loss, however, was different in the two groups.

The most striking effect of growth hormone seen in these studies was the intense mobilization of lipid to liver tissue. It may be noted in Figure 2 that although there was an actual loss of about 20% of the total lipid from the liver in the untreated intact group during the fast, this was counteracted to a striking degree by the administration of growth hormone. The scale here is 8 times reduced over

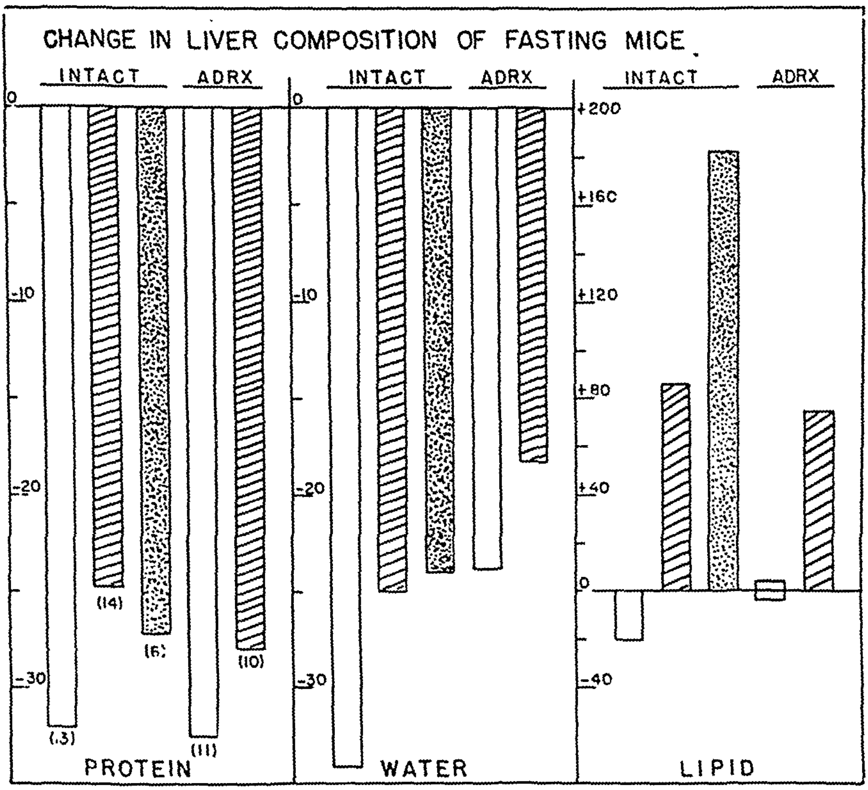


FIG. 2. The length of the bars represents per cent change in liver constituents of fasted mice related to the tissue composition of fed controls taken as a zero baseline. Comparison is made between intact fasted *vs.* intact fed control, and adrenalectomized fasted, *vs.* adrenalectomized fed, animals. (*v.*, Results, section 1.). The absolute amounts of each constituent present at the start of the fast are inferred for each group from the liver weight: body weight ratio and liver composition found by simultaneous analysis of fed controls. The open bars represent untreated, fasted animals, the cross-hatched bars, animals receiving 200 micrograms of growth hormone, and the stippled bars, those to which the 4 mgm. dose of hormone was administered. The first three bars in each section refer to intact animals, the last group in each section represents adrenalectomized mice. The number of ani[illegible] the average in each case is indicated by the number enclosed in [illegible] values indicate losses, positive values, gains.

that used for protein and water in order to show the intense lipid infiltration produced; the 4 mgm. dose increased the total lipid by almost 200%. It is possible that these values were observed at a time beyond the maximum lipid accumulation (cf. Steppuhn, 1934; Campbell, 1938; and Hodge et al., 1948). Even in fasting, adrenalectomized animals which, in confirmation of MacKay and Barnes (1937a) were unable to mobilize lipid to the liver, the administration of growth hormone produced a similar increase in liver lipid.

Table 2 demonstrates the influence of growth hormone on liver weight of fasting mice. It will be observed that liver wet weight per 100 gm. final body weight was significantly greater in growth hormone-treated animals, both intact and adrenalectomized, than in their respective fasting controls, despite the fact that the adrenalectomized mice began the fast at a lower initial level. In addition, fat-free liver weight, and total dry, fat-free weight were with one exception significantly greater in growth hormone-treated, fasted mice than in their fasted controls, demonstrating again the relative retention of nitrogen even when computed on a fat-free, and on a water-free basis. Thus, notwithstanding the fact that nitrogen *concentration* was reduced because of the tremendous lipid infiltration, the relatively greater liver mass in the growth hormone-treated animals prevented the nitrogen from reaching as low levels as those seen in fasting alone. This is depicted in Table 3, which shows the greater amounts of protein and water *as well as* lipid in the livers of growth

TABLE 3. THE INFLUENCE OF GROWTH HORMONE ON LIVER COMPOSITION IN MALE CBA MICE DURING FASTING†

| Group | No. of Animals | Liver Nitrogen, % | Total Liver Protein (gm.) | Liver Water, % | Total Liver Water, (gm.) | Liver Lipid, % | Total Liver Lipid, (gm.) |
|---|---|---|---|---|---|---|---|
| *Intact* | | | | | | | |
| Fed | 8 | 3.48 ± 0.045 | 0.296 ± 0.010 | 69.7 ± 0.39 | 0.955 ± 0.045 | 7.52 ± 0.27 | 0.103 ± 0.006 |
| Fasted 48 hrs. | 13 | 3.64 0.045 ± | 0.196 ± 0.007 | 68.3 ± 0.59 | 0.604 ± 0.030 | 8.85 ± 0.64 | 0.079 ± 0.008 |
| Fasted 48 hrs. + Growth Hormone, 200 $\mu$gm. | 14 | 3.16 ± 0.078* | 0.218 ± 0.006* | 63.0 ± 1.18* | 0.697 ± 0.020* | 16.4 ± 1.26* | 0.187 ± 0.020* |
| Fasted 48 hrs. + Growth Hormone, 4 mgm. | 6 | 2.77 ± 0.18* | 0.212 ± 0.005 | 58.0 ± 2.19* | 0.712 ± 0.020* | 23.0 ± 3.0* | 0.295 ± 0.056* |
| *Adrenalectomized* | | | | | | | |
| Fed | 13 | 3.61 ± 0.062 | 0.252 ± 0.007 | 70.6 ± 0.40 | 0.792 ± 0.030 | 7.51 ± 0.32 | 0.083 ± 0.003 |
| Fasted 48 hrs. (+ saline) | 11 | 3.26 ± 0.08 | 0.181 ± 0.006 | 71.5 ± 0.70 | 0.636 ± 0.016 | 9.78 ± 0.82 | 0.088 ± 0.010 |
| Fasted 48 hrs. + Growth Hormone 200 $\mu$gm. | 10 | 2.99 ± 0.07* | 0.192 ± 0.005 | 66.0 ± 1.55* | 0.679 ± 0.021* | 15.21 ± 1.85* | 0.157 ± 0.019* |

† Deviations shown are standard errors.
* The starred values are significantly different from the respective fasted control figures.

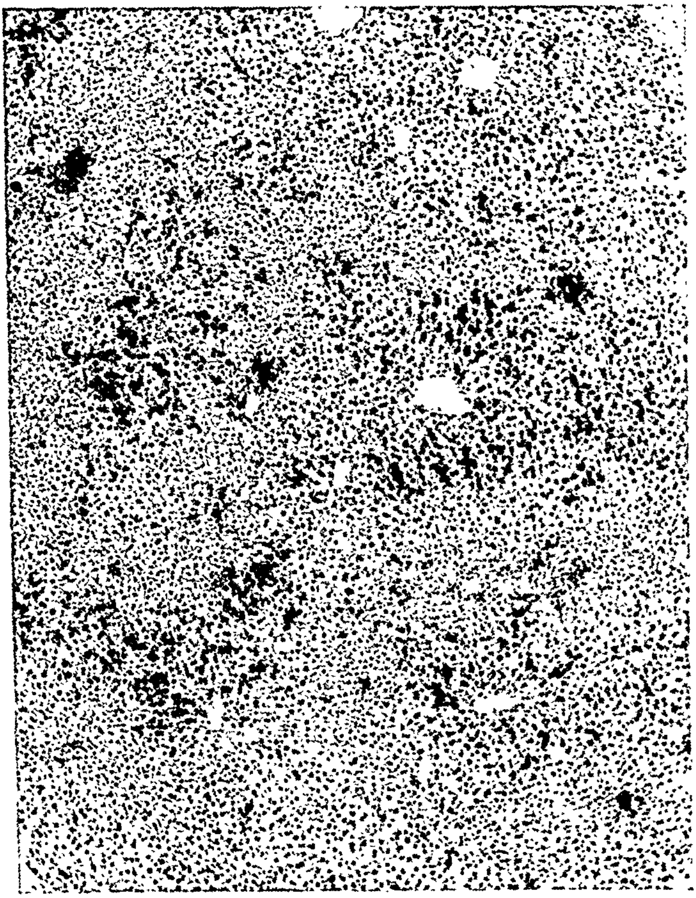

FIG. 3. Photomicrograph of a section of the liver of a mouse which had received 200 micrograms of growth hormone during a 48-hour fast. Hematoxylin-eosin stain; magnification ×65; lipid content, 16.8% of the fresh weight. Note preservation of architecture, light-staining (vacuolated) periportal areas, considerable variation in nuclear size, and absence of necrosis.

hormone-treated animals compared with fasting controls. These data are statistically significant where indicated.

b. *Histological appearance.*[4]

In spite of intense hepatic lipid infiltration, there was no microscopic evidence of any essential disturbance of lobular architecture in any of the series of livers from fasting animals treated with growth hormone. No sign of necrosis was observed even with lipid concentrations up to 40% of the wet weight of the liver, although some variation in nuclear size has been noted. A typical photomicrograph is reproduced in Fig. 3. Sudan IV and Sudan Black stains indicated a periportal lipid distribution, with sparing of the central areas in most

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[4] Dr. Henry Bunting of the Department of Pathology and Dr. Elijah Adams of the Department of Physiological Chemistry, Yale University, kindly aided in the interpretation of histological sections of liver and other tissues.

cases. The intensity of Sudan staining was well correlated with lipid content found by chemical analysis and progressive increases in staining capacity were observed: from slight, seen in livers of control fasted series (8–10% total lipid), through moderate, with the 200 microgram dose of growth hormone (15–16% lipid), to intense, resulting from the administration of 4 mgm. growth hormone during the fast (average 23% lipid). Livers from fed animals, with an average lipid content of 7.5% of the wet weight of the liver, showed no Sudan staining. The above remarks on liver lipid distribution under the influence of growth hormone in fasting mice also apply in general to the adrenalectomized group, in which, however, sparing of central areas was less pronounced. Preliminary evidence obtained from fractionation studies indicated that the bulk of the excess liver lipid accumulated by growth hormone-treated, fasted animals was neutral fat.

3. *The influence of growth hormone on the composition of weight loss of fasting mice.*

Figure 4 demonstrates the effect of growth hormone on the composition of weight loss of the residual carcass of growth hormone-treated *vs.* control fasted mice. These data are plotted in terms of grams of each constituent lost per 100 gm. initial body weight. It will be observed from the figure and also from Table 4 that a distinct and significant reduction in body weight loss due to fasting was brought about by both doses of growth hormone in intact animals

TABLE 4. THE INFLUENCE OF GROWTH HORMONE ON THE COMPOSITION OF THE WEIGHT LOSS OF FASTING CBA MICE

| Group | No. of Mice | Orig. B.W. (gm.) | Wt. Loss (gm.)[1] | "p" Value[2] | Ave. Final N (%)[3] | Wt. Loss as Prot. (gm.)[4] | Ave. Final $H_2O$ (%)[3] | Wt. Loss as $H_2O$ (gm.)[4] | Ave. Final Lipid (%)[3] | Wt. Loss as Lipid (gm.)[4] | Ratio Prot.: $H_2O$ Loss | Ratio Prot.: Lipid Loss |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Intact* | | | | | | | | | | | | |
| Fed | 8 | 22.4 | — | — | 3.15 | — | 66.3 | — | 9.95 | — | — | — |
| Fasted 48 hrs. | 13 | 21.8 | 5.5± 0.26 | — | 3.46 | 1.13 | 68.5 | 3.71 | 4.74 | 0.40 | 1:3.26 | 1:0.354 |
| Fasted+Growth Hormone 200 μgm | 14 | 22.0 | 4.6 ± 0.17 | <0.01 | 3.45 | 0.95 | 69.0 | 3.12 | 5.09 | 0.35 | 1:3.30 | 1:0.369 |
| Fasted+Growth Hormone, 4 mgm. | 6 | 22.2 | 4.2 ± 0.26 | <0.01 | 3.28 | 0.85 | 68.1 | 2.82 | 4.98 | 0.32 | 1:3.34 | 1:0.376 |
| *Adrenalectomized* | | | | | | | | | | | | |
| Fed | 9 | 20.6 | — | — | 3.16 | — | 67.6 | — | — | — | — | — |
| Fasted 48 hrs. (+ saline) | 13 | 21.8 | 2.4 ± 0.23 | — | 3.16 | 0.48 | 71.3 | 1.67 | — | — | 1:3.50 | — |
| Fasted 48 hrs +Growth Hormone 200 μgm. | 9 | 21.7 | 2.0 ± 0.27 | <0.2 | 3.23 | 0.41 | 71.6 | 1.39 | — | — | 1:3.42 | — |

[1] Standard errors are shown.
[2] Between each group given growth hormone and the fasted control.
[3] All analyses refer to whole carcass minus liver, gut and a constant proportion of lymphoid tissue.
[4] The contribution of each constituent to the weight loss is calculated by multiplying the average composition between initial and final levels by the observed weight loss.

("p" value <0.01). This was due, in the main, to reduced loss of protein and water. The differences in carcass lipid loss between control and growth hormone-treated animals are probably not significant because of the greater variability of initial carcass fat depots. However, Table 4 reveals that a somewhat greater amount of carcass lipid is lost per unit of protein loss in the growth hormone-treated animals.

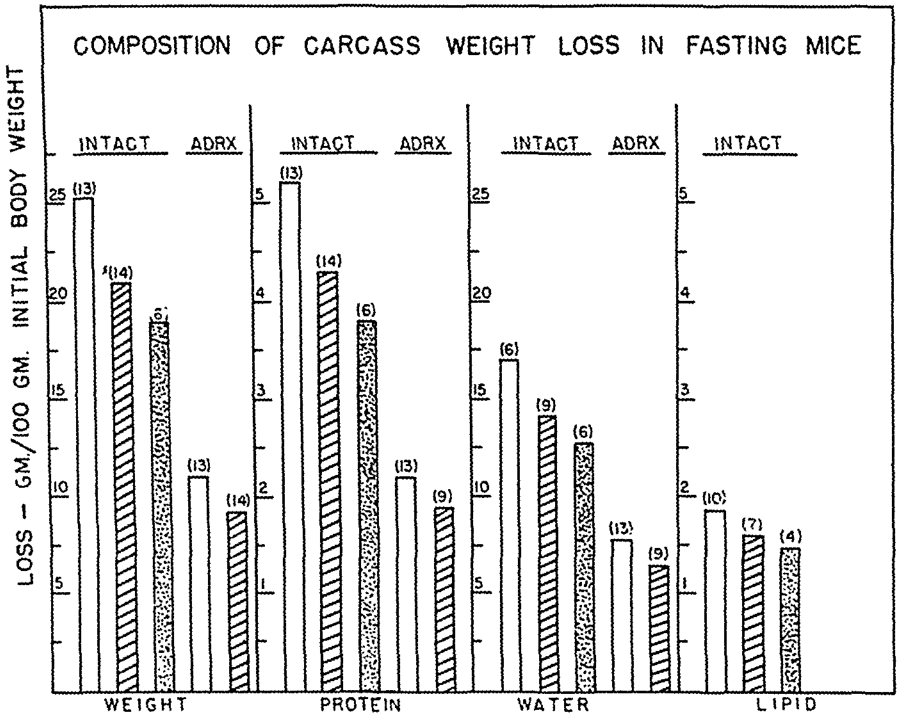


FIG. 4. The length of the bars represents loss of each constituent in gm./100 gm. initial body weight. The contribution of each constituent to the weight loss is calculated from the average composition during the fasting period, between initial (appropriate control) and final (observed) levels, and multiplying by observed weight loss. The open bars represent untreated, fasted animals, the cross-hatched bars, animals receiving 200 micrograms of growth hormone, and the stippled bars, those to which the 4 mgm. dose of hormone was administered. The first three bars in each section refer to intact animals, the last group in each section represents adrenalectomized mice. The number of animals contributing to the average in each case is indicated by the number enclosed in parentheses.

In adrenalectomized animals treated with growth hormone, there appeared to be a relative retention of nitrogen and water in the carcass, although the magnitude of the changes is not statistically significant (Table 4, Figure 4).

4. *The composition of lymphoid tissue weight change in fasting mice.*

In Figure 5 and Table 5 are indicated the fasting changes in lymphoid tissue composition. It will be observed that a profound fasting

involution of lymphoid tissue occurred in intact, untreated animals (cf. White and Dougherty, 1947). This was augmented, probably as a result of non-specific stress, by the large dose of growth hormone (4 mgm.), but this augmentation was of limited significance ($p = 0.1$). The lower dose of growth hormone was subsequently employed because, although it did not accelerate the fasting involution of lymphoid

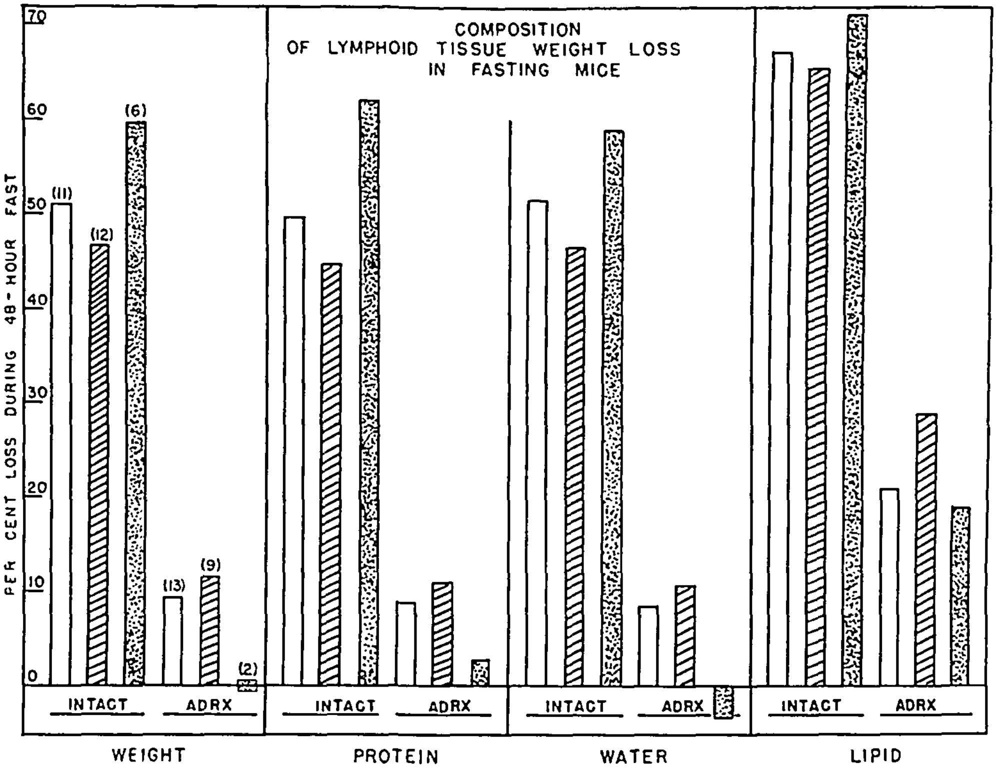


FIG. 5. The length of the bars represents per cent loss in lymphoid tissue constituents of fasted mice related to the tissue composition of fed controls taken as a zero baseline. Comparison is made between intact fasted *vs.* intact fed control, and adrenalectomized fasted *vs.* adrenalectomized fed, animals. (*v.*, Results, section 1.). The absolute amounts of each constituent present at the start of the fast are inferred for each group from the lymphoid tissue weight: body weight ratio and composition found by simultaneous analysis of fed controls. The open bars represent untreated, fasted animals, the cross-hatched bars, animals receiving 200 micrograms of growth hormone, and the stippled bars, those to which the 4 mgm. dose of hormone was administered. The first three bars in each section refer to intact, the second three to adrenalectomized, animals. The number of animals contributing to the average in each case is indicated by the number enclosed in parentheses. Values falling on the baseline indicate no change from, and values below the baseline, gain over, the respective control level.

tissue in the intact animal, it was nevertheless active in producing other effects (cf., sections 2, 3).

Protein and water were lost from lymphoid tissue in similar proportions during fasting, regardless of growth hormone administration, but lipid appeared to be further reduced, relative to total lymphoid tissue loss. This may indicate a selective mobilization of lipid beyond that lost as a consequence of dissolution of individual cells.

TABLE 5. THE COMPOSITION OF LYMPHOID TISSUE IN FASTING MICE±

| Group | No. of Mice | Wet wt. Lymphoid Tissue (gm./100 gm. B.W.) | Loss in Total Lymphoid Tissue Wt. (%) | Percentage Composition | | |
|---|---|---|---|---|---|---|
| | | | | Nitrogen | Water | Lipid |
| *Intact* | | | | | | |
| Fed | 8 | 0.870 ± 0.039 | — | 2.65 ± 0.05 | 77.7 ± 0.6 | 7.69 |
| Fasted 48 hrs. | 11 | 0.555 ± 0.042 | 51.0 | 2.75 ± 0.04 | 78.2 ± 1.0 | 5.20 |
| Fasted 48 hrs. + 200 μgm. Growth Hormone | 12 | 0.587 ± 0.023 | 46.6 | 2.75 ± 0.03 | 77.9 ± 0.2 | 4.9† |
| Fasted 48 hrs. + 4 mgm. Growth Hormone | 6 | 0.444 ± 0.040[1] | 59.5 | 2.43 ± 0.09* | 77.0 ± 0.5 | 5.37 |
| *Adrenalectomized* | | | | | | |
| Fed | 9 | 1.179 ± 0.088 | — | 2.64 ± 0.02 | 78.9 ± 0.3 | 4.6† |
| Fasted 48 hrs. (+saline) | 13 | 1.202 ± 0.065 | 9.3 | 2.65 ± 0.04 | 79.4 ± 0.2 | 4.0† |
| Fasted 48 hrs. + 200 μgm. Growth Hormone | 9 | 1.148 ± 0.064 | 11.7 | 2.65 ± 0.03 | 79.7 ± 0.2 | 3.7† |
| Fasted 48 hrs. + 4 mgm. Growth Hormone | 2 | 1.225 | 0 | 2.52 | 80.5 | 3.7† |

± Deviations shown are standard errors.
† By difference.
* The starred values are significantly different from the respective fasted control figures.
[1] Of limited significance: $p = 0.1$.

This disproportionately greater loss of lymphoid tissue lipid, compared to losses in other constitutents, appeared to occur also in adrenalectomized animals during fasting, even though there was no significant loss in nitrogen or water. Actually, the total lymphoid tissue weights, per 100 gm. body weight, were not significantly affected by fasting, or by either of the two growth hormone doses (Table 5.) in the adrenalectomized group. When, on the other hand, lymphoid tissue of fasting adrenalectomized mice is compared to the corresponding structures of fasting intact animals, ample evidence of growth after adrenalectomy is observed from these data.

## DISCUSSION

The catabolic influence of the adrenal cortical steroids, as evidenced by their capacity to accelerate the rate of nitrogen excretion (cf. review by White, 1948), is generally acknowledged. In the adrenalectomized, fasting rat, urinary nitrogen falls to a low level, suggesting a retardation of the rate of tissue catabolism (Evans, 1936; Harrison and Long, 1940). This is further exemplified in the reduction in nitrogen loss from carcass and lymphoid tissue reported by White and Dougherty (1947) in fasting, adrenalectomized mice. The results re-

ported in the present investigation indicate that even this low rate of tissue breakdown can be further suppressed or counteracted by the administration of growth hormone (or of anterior pituitary extracts possessing growth-promoting activity, cf., Harrison and Long, 1940), and constitute additional evidence of the opposing actions of growth and adrenal cortical hormones (cf., Marx et al., 1943). In the present investigation, growth hormone exerted its nitrogen-sparing effect in the adrenalectomized fasting mouse primarily in the liver.

The administration of growth hormone to the fasting mouse resulted in a diminished rate of nitrogen and water loss, associated with an apparent acceleration of fat metabolism. These observations are analagous to the effects noted in the fed animal by Lee and Schaffer (1934). The weight gain of fed rats treated with semi-purified growth-promoting extracts was composed of an increased proportion of nitrogen, water, and ash, and a decreased concentration of fat, as compared to control, pair-fed litter mates. It was deduced from the latter studies that chemical maturation, according to the concepts of Moulton (1923), was delayed by treatment of the animals with anterior pituitary extract.

The shift to an increased rate of fat oxidation, inferred from the earlier work with crude anterior pituitary extracts (cf., Schäfer (1931), Wadehn (1932), Gaebler (1933), Lee and Schaffer (1934), Steppuhn (1934), Best and Campbell (1936), Harrison and Long (1940), and others), was reflected in the present studies primarily by the metabolic events occurring in the liver. The slight hepatic fatty infiltration which has been observed as a result of fasting alone, was strikingly exaggerated by the administration of growth hormone to the intact animal. This treatment resulted in exceedingly fatty livers, which were, however, histologically free from necrosis or inflammatory processes. Downs (1930) and Best and Campbell (1936) described liver necrosis following injection of crude anterior pituitary extracts; the latter authors also observed fatty infiltration of the liver. Purified growth hormone has also been reported recently by Bennett et al. (1948) to possess ketogenic properties. Ketosis was prevented by removal of the liver in the latter studies, as well as in the earlier experiments of Mirsky (1936) who used crude extracts. It was further observed by Shipley (1944) that ketogenesis by rat liver *in vitro* was accentuated by anterior pituitary extract. These studies indicated the importance of the liver in mediating the accelerated fat metabolism. It is possible that the liver may be essential for other manifestations of growth hormone activity. Thus, Frame and Russell (1946) found that whole anterior pituitary extract, presumably rich in growth-promoting activity, decreased the blood amino acid content of normal rats, but was without effect in the eviscerated animal. The liver has also been directly implicated in the earliest observed effects of anterior pituitary extracts i.e., reductions in liver non-protein

nitrogen and urea (Lee and Schaffer, 1934), and in glutathione (Gregory and Goss, 1939). It seems probable from these data that the liver may be a principal site of action of growth hormone (cf., Lee, 1936).

The present studies reveal that purified growth hormone is capable of causing an increase in liver fat in the fasting, adrenalectomized animal notwithstanding the impairment of fasting lipid mobilization to the liver in the absence of the adrenals (cf., also MacKay and Barnes, 1937a). It had been shown by Fry (1937) and MacKay and Barnes (1937a) that crude anterior pituitary extracts failed to induce fatty liver in the adrenalectomized rat. Adrenalectomy also prevented the fatty infiltration of the liver which usually accompanies pancreatectomy (Long and Lukens, 1936), diminished the deposition of liver fat following partial hepatectomy (MacKay and Carne, 1938; Berman et al., 1947), and reduced the ketonuria of fasting rats after anterior pituitary extract administration (Fry, 1936; MacKay and Barnes, 1937b). It is apparent, however, that under the influence of the more highly purified growth hormone fraction used in the present investigation, fat mobilization to the liver was independent of the adrenal. The ketogenic effects of purified growth hormone have also been observed in the absence of the adrenal by Bennett et al., (1948). The available evidence suggests that the shift to accelerated fat metabolism promoted by growth hormone is not necessarily adrenal-mediated. The interpretation of the earlier data demonstrating that the presence of the adrenal is essential for liver lipid accumulation with crude anterior pituitary extracts is made difficult by the complex nature of the extracts used.

In view of the profound effects of growth hormone on the metabolic pattern of the fasting animal, it is somewhat surprising that lymphoid tissue appeared to be independent of its influence. Neither the composition nor the weight of the lymphoid structures of the intact, fasted animal was altered by growth hormone administration. The accentuated involution of lymphoid tissue produced by very large doses of this hormone was probably due to a non-specific augmentation of pituitary-adrenal cortical secretion. Marx, Simpson, Reinhardt and Evans (1942), however, reported some increase in thymus weight beyond that expected as a result of body weight gain in fed rats injected chronically with relatively pure growth hormone.

The increased mass of lymphoid structures seen following adrenalectomy appeared to fulfill the biochemical criteria of true growth i.e., the deposition of relatively larger amounts of nitrogen and water and a lower proportion of fat as compared to the adult structures. This growth occurs notwithstanding the obvious drain on the energetic resources of the animal during fasting, and is further evidence of the decreased rate of utilization of lymphoid tissue nitrogen in the ab-

sence of the adrenal steroids (White and Dougherty, 1947). The data appear to suggest, however, that the lipid of lymphoid tissue is a more labile component, and may be available to the fasting animal even in the absence of the adrenals. The growth of lymphoid tissue following adrenalectomy could not be potentiated by growth hormone in the fasting animal. Thus, the rôle of the adrenal in regulating the structure and function of lymphoid tissue gains emphasis from the failure to influence this tissue with growth hormone, which, in general, has a nitrogen-sparing effect in the fasting animal.

The metabolic effects of anterior pituitary extracts have been reviewed by Houssay (1942) and Long (1942, 1943), and, more recently, for growth-promoting fractions of a greater degree of purity, by Marx and Evans (1944) and Li and Evans (1947). There appear to be several possible mechanisms by which growth hormone may act to produce the metabolic changes observed in the present investigation and in other studies. The evidence is consistent with the alternatives a), that growth hormone inhibits the rate at which the animal is able to mobilize or utilize protein and is therefore compelled to burn a greater proportion of fat to supply its energy needs, or, b), that growth hormone accelerates the rate at which the animal can mobilize fat—i.e., that it burns fat preferentially, and consequently less protein is required as an energy source. A fat mobilizing factor in the cruder pituitary extracts was termed "lipoitrin" by Raab (1934). The magnitude and early onset (Campbell, 1938) of fat mobilization following anterior pituitary extract injections suggest that the effects of growth hormone on the nitrogenous constituents of liver and other tissues may be secondary. The influence of anterior pituitary extract in promoting fat metabolism has been advanced by others (Gaebler, 1933; Lee and Schaffer, 1934; and Long, 1943) as a possible explanation for the mechanism by which protein could be spared. Growth hormone may produce at least a portion of its effects by reducing total metabolism (Kleiber and Cole, 1939), although this was not seen in earlier experiments by Gaebler (1933, 1935), who reported a large calorigenic response concomitant with a fall in R.Q. in dogs treated with crude anterior pituitary extracts. Whatever the primary mechanism of growth hormone action may be, it appears to be mediated by, or require the participation of, the liver.

In considering the economy of the fasting animal, it is possible that growth hormone production is stimulated by fasting, as appears to be the case for adrenotrophin. This suggestion is supoprted by the observation that the untreated, fasting animal burns a larger proportion of lipid than does the fed subject. The latent period during which the urinary nitrogen falls progressively to a minimal level as fasting proceeds may be due to the time required for the activity of endogenous growth hormone to be manifested, and for adrenal exhaustion and consequent decline of the catabolic effects of the adrenal cortical steroids.

## SUMMARY

The influence of a purified growth hormone preparation on fasting metabolism of intact and adrenalectomized mice has been investigated. Doses of 200 micrograms, administered during a 48-hour fasting period, caused a depression of the rate of nitrogen and water loss from liver and carcass of these animals. This relative sparing of nitrogen was accompanied by an acceleration of fat metabolism as evidenced by intense fatty infiltration of the livers of fasting animals treated with growth hormone. The accumulation of liver lipid as a result of growth hormone injection was independent of the presence of the adrenals. Histological examination did not reveal evidence of liver damage. The data support the suggestion that the lipid mobilizing action of growth hormone may be integrated with its nitrogen sparing effect.

The involution of lymphoid tissue in the fasting mouse was independent of the influence of growth hormone, except at very large doses (4 mgm./48 hours), which accentuated this phenomenon in intact mice. The increase in mass of lymphoid tissue following adrenalectomy was not potentiated by growth hormone administration during fasting. Analysis of the composition of the lymphoid tissue weight gain following adrenalectomy yielded evidence that this enlargement conformed to the criteria of true growth.



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Miss Jeanette Opsahl and Miss Rita Quan contributed valuable assistance in the analytical procedures.


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# SEASONAL CHANGES IN THE TESTES, EPIDIDYMIDES AND SEMINAL VESICLES OF DEER INVESTIGATED BY HISTOCHEMICAL METHODS[1]

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IT WAS shown some years ago that the testes, epididymides and seminal vesicles of Virginia deer exhibit seasonal changes in respect to their weights and histological appearances (Wislocki, '43). In the latitude of New England, they increase in weight and reach maximal activity in the fall (October, November), followed by a marked decline in weight and suppression of secretory activity and spermatogenesis in the spring (May, June).

Since the former report was published, the internal sex organs of male deer have been reinvestigated in this laboratory by a variety of histochemical methods for lipids (including steroids), nucleoproteins, acid mucopolysaccharides, glycogen and phosphatases. The present paper describes these reactions in the testes, epididymides and seminal vesicles of Virginia and Japanese deer, comparing the results in the active and inactive states.

## MATERIALS AND METHOD

The material consists of the testes and seminal vesicles of 4 Virginia deer (Odocoileus virginianus borealis) and 2 Japanese or Sika deer (Cervus nippon).[2] The 4 Virginia deer, from a healthy herd on free range, were obtained on June 9 and 14, October 19 and November 30. The 2 Japanese deer, from a small captive herd, were taken on June 13 and November 13.

For the staining of lipids, pieces of tissue were fixed in 10% neutral formalin. Frozen sections were stained in either sudan III (sat. sol. in 70% alcohol with 1% pot. hydrox. added) or sudan black B (sat. sol. in 70% alcohol) for 7 minutes and then mounted in glycerine jelly.

For the demonstration of steroid substances, pieces of testes were fixed in 10% neutral formalin. Unstained, frozen sections of these blocks were examined in ultraviolet light for natural fluorescence and with the polarizing

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microscope for birefringence, while other sections were stained with sudan III or sudan black B and with Schiff's plasmal reagent (the plasmal reaction of Feulgen and Voit). Other sections were extracted with acetone at room temperature before carrying out the above procedures.

No single method is available for identifying ketosteroids in tissue sections, but by the use of a combination of methods, the sum of which characterizes ketosteroids (but no other known type of compound) they can be identified with some assurrance (Bennett, '40; Dempsey and Wislocki, '46). Since steroids are soluble in acetone at room temperature, the other methods used will be negative following extraction of the sections in that solvent. The steroids, along with other lipids, are sudanophilic and hence will be stained with sudan dyes. Because of their ketone groups, the ketosteroids will recolorize leucofuchsin (Schiff's reagent) to produce a purple color (the "plasmal reaction" of Feulgen and Voit). Unsaturated steroids exhibit greenish or yellowish autofluorescence when illuminated by ultraviolet light, and are visible in cells as birefringent spherocrystals.

For the investigation of cytoplasmic basophilia, blocks of tissue were fixed in Zenker's fluid and the deparaffinized sections were stained with eosin and methylene blue or with toluidin blue ($\frac{1}{2}\%$ aqueous sol.). The Feulgen reaction for desoxyribonucleic acid was carried out by the standard technique after fixation in Zenker's fluid.

Acid mucopolysaccharides were sought for by means of their metachromatic staining. Pieces of tissue were fixed in 4% basic lead acetate and deparaffinized sections were stained in a $\frac{1}{2}\%$ aqueous solution of toluidin blue for 30 minutes (Holmgren, '40; Wislocki, Bunting and Dempsey, '47).

Glycogen was sought for in pieces of tissue fixed in Rossman's fluid (abs. alc. sat. with picric acid, 90 cc.; formaldehyde (40%), 10 cc.). Deparaffinized sections were stained by both the Bauer-Feulgen and periodic acid-Schiff methods. Control sections were exposed to saliva for 30 minutes at room temperature before staining. The periodic acid-Schiff method was applied according to the directions of McManus ('46). The deparaffinized sections are placed in a 1% solution of periodic acid for 5 minutes; they are then washed and stained for 10 to 15 minutes in Schiff's reagent, transferred directly to 2 changes of sulphurous acid for 5 minutes each, then cleared and mounted in balsam.

Besides glycogen, the periodic acid-Schiff method stains other substances and structures, including mucus, cartilage matrix, collagenous fibers, basement membranes, fibrin and the elastica interna of blood vessels (Lillie et al., '47; Wislocki and Dempsey, '48). Besides glycogen, the Bauer-Feulgen reaction which employs chromic acid in a way similar to periodic acid stains mucus, cartilage matrix and elastica interna (Wislocki, Bunting and Dempsey, '47). Unlike the staining of glycogen, these latter reactions are not prevented by previous treatment of the sections with saliva. Staining by these 2 methods depends probably on the oxidation of polysaccharide complexes in various glycoproteins by periodic or chromic acid, with the result that aldehydes are formed. The latter are revealed by their reaction with the leucofuchsin in Schiff's reagent.

Alkaline phosphatase was investigated by the method of Gomori ('41a) as modified by Dempsey and Deane ('46) utilizing sodium glycerophosphate, fructose diphosphate and yeast nucleic acid as substrates. Pieces of tissue

were fixed in chilled 80% alcohol and the sections incubated at pH 9.4 for 3 and 6 hours. The sections were counterstained with Mayer's paracarmine.

Acid phosphatase was demonstrated by Gomori's method ('41b) in tissues fixed in chilled acetone and incubated in glycerophosphate at pH 4.7 for 24 and 48 hours. The sections were counterstained with Mayer's paracarmine.

Besides the use of these more specific histochemical methods occasional sections were stained by other techniques. The latter included staining by Masson's triacid mixture (ponceau-acid fuchsin, orange G and light green), hematoxylin and eosin, Heidenhain's iron-hematoxylin method, Bodian's protargol methods as modified by Dawson and Barnett ('44) and Pap's silver impregnation method for reticulum (Pap, '29; Mitchell and Wislocki, '44).

## TESTIS

The testes of deer killed in New England are active and large at the time of the rutting season in the fall and are small and atrophic in the spring (Wislocki, '43). The present investigation compares the cytology and histochemistry of the testes of several Virginia and Japanese deer at these 2 periods when the seasonal contrasts are maximal. The intermediate phases of the intervening months have not been followed.

### THE INTERSTITIAL TISSUE

The interstitial tissue of the testes is more voluminous in the rutting season than in the sexually quiescent months of the spring. In the fall the cells are large and numerous and their cytoplasm is abundantly filled with sudanophilic lipid droplets (figs. 1 and 10). Sections stained by Schiff's reagent reveal an intense plasmal reaction (figs. 4 and 11) which appears to coincide with the sudanophil droplets (cf. figs. 10 and 11). Observed under the polarizing microscope, many birefringent droplets are encountered and when examined for fluorescence in ultraviolet light the interstitial cells emit a yellowish autofluorescence. These reactions are lost after preliminary extraction of the frozen sections with acetone.

In June testes the cells are smaller and fewer in number and the sudanophilic droplets are diminished (fig. 12). The Schiff reaction, birefringence and autofluorescence are correspondingly reduced. The above observations indicate not only that the interstitial tissue is the site of formation of ketosteroids but that the quantity present is much greater in the fall at the time of rut than during the period of sexual inactivity.

In the course of preparation of paraffin sections of the testes the fat droplets are ordinarily dissolved out, giving a delicate foamy appearance to the cytoplasm of the interstitial cells. This is apparent in sections fixed in Zenker's fluid and subsequently stained with eosin and methylene blue or with Masson's triacid mixture.

In the fall testes, fixed in Zenker's fluid and stained with eosin and methylene blue, the cytoplasm of the interstitial cells stains a

lavender color with superimposed delicate basophilic stippling. In June the shrunken cytoplasm of the interstitial cells is paler in color and shows practically no cytoplasmic basophilia.

With toluidin blue, after fixation in 4% basic lead acetate, metachromasia has not been encountered in the interstitial tissue at either time of the year, an observation indicating the absence of any acid mucopolysaccharides.

Crystals have not been encountered in the interstitial cells of deer, although they were sought for in sections stained with Heidenhain's iron-hematoxylin, eosin and methylene blue and Masson's triacid stain.

By the Bauer-Feulgen and periodic acid-Schiff procedures glycogen is not demonstrable in the interstitial cells, but it occurs with regularity in the walls of the arteries and arterioles. By the periodic acid-Schiff method the basement membranes surrounding the seminiferous tubules and collagen in general are intensely colored (fig. 3), but, since this staining is not prevented by preliminary exposure of the sections to saliva, it is not due to glycogen. Similarly, in June testes glycogen is present in the walls of the blood vessels but is absent from the interstitial cells. With the periodic acid-Schiff method the fibrous stroma of June testes is especially deeply stained, a result attributable to the relative increase in density of the collagen in the shrunken organ.

The cytoplasm of the interstitial cells in the fall testes contains little alkaline phosphatase, whereas the basement membranes enclosing the seminiferous tubules and the walls of the interstitial arteries and arterioles are intensely stained (figs. 5 and 6). With fructose diphosphate as substrate the cytoplasm of the interstitial cells is more distinctly stained (fig. 5) than after the use of yeast nucleic acid (fig. 6). In the interstitial tissue of June testes, on the contrary,

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Explanation of Figures

1. Frozen section of a formalin-fixed testis of a Virginia deer (Nov. 30) stained with sudan III for 7 minutes. Observe the staining of the interstitial tissue and the seminiferous tubules. ×160.

2. Frozen section of formalin-fixed testis of a Japanese deer (Nov. 13) stained with sudan black B for 7 minutes. Notice the stained lipid droplets in the elements of the seminiferous tubule and compare with figure 1. The sperm heads, seen in profile, are accentuated by sudan dyes. ×70 ocular, ×90 objective.

3. Section of a testis of a Virginia deer (Oct. 19) fixed in Rossman's fluid and stained by McManus' periodic acid-Schiff procedure. Observe the staining of the basement membranes enclosing the seminiferous tubules and of the walls of the blood vessels of the interstitial tissue. The interstitial cells are unstained. Notice the relatively faintly stained spermatids in the 2 tubules in the lower half of the photograph and compare them with the cells shown in figure 15. In the tubule visible in the upper half of the photograph, observe the more deeply stained spermatozoa and compare them with figure 16. ×260.

4. Frozen section of a formalin-fixed testis of a Virginia deer (Nov. 30) stained with Schiff's reagent illustrating the prominent plasmal reaction of the interstitial tissue. Compare this photograph with a drawing of the interstitial tissue at higher magnification (fig. 11). ×160.

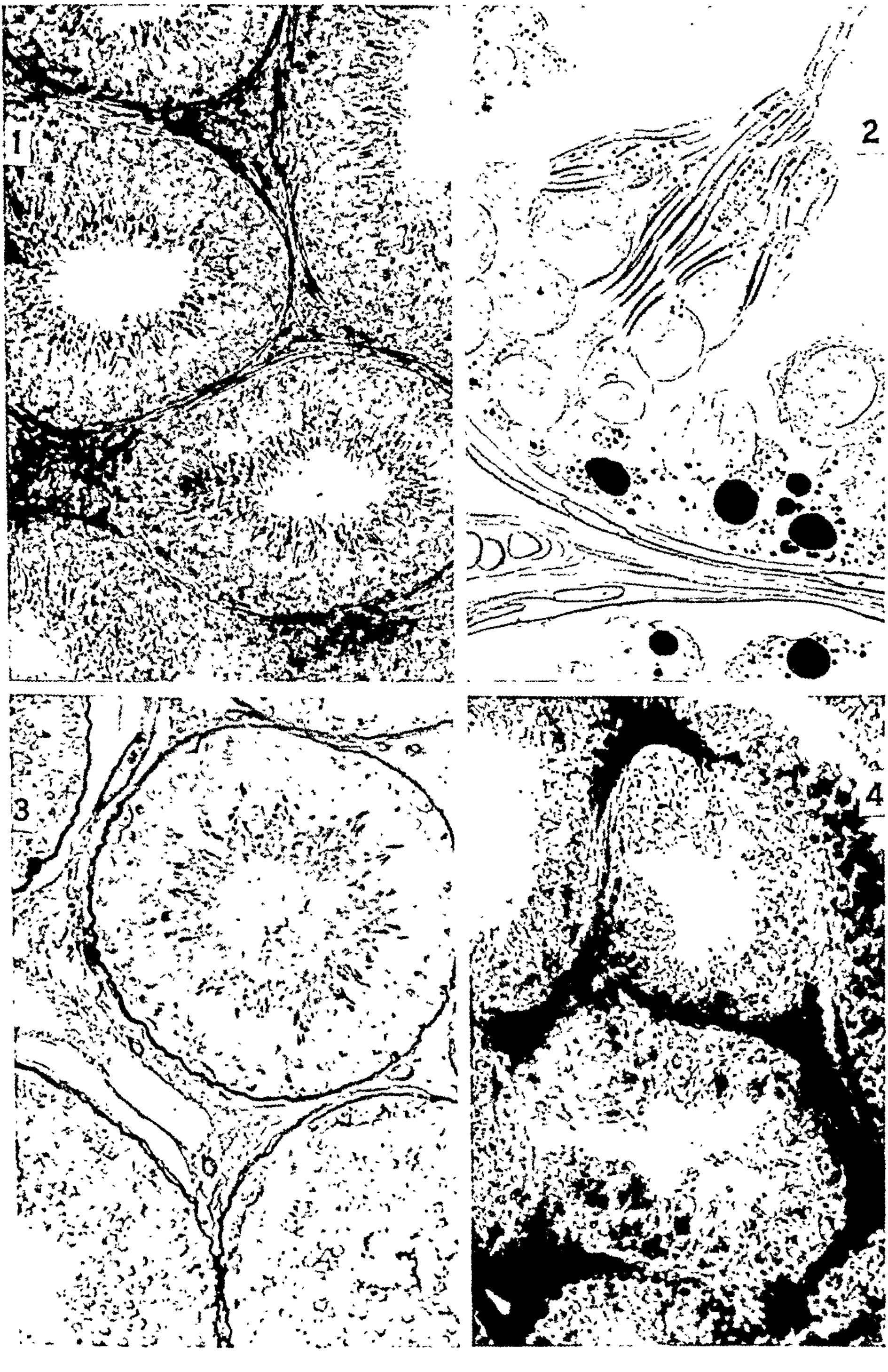


Plate 1

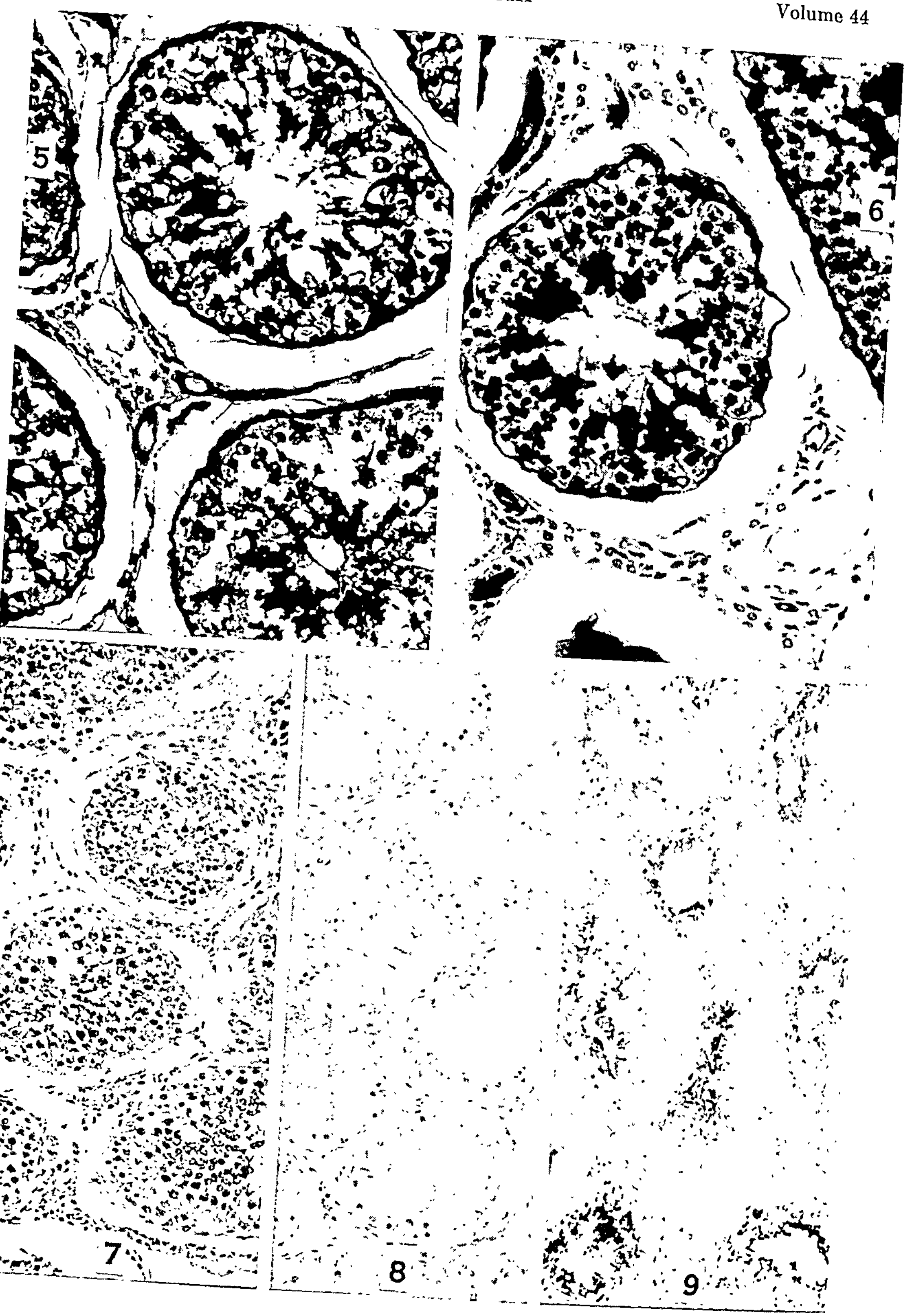


PLATE 2

alkaline phosphatase is practically absent (fig. 9). It should be noted that the staining of the nuclei of the interstitial cells registered in photographs 5, 6 and 9 is due mainly to counterstaining by paracarmine and not to the presence of alkaline phosphatase.

Acid phosphatase is present in slight amounts in the interstitial tissue of fall testes (fig. 7). Differing from alkaline phosphatase, it is present principally in the cell nuclei and does not occur in either the arterial walls or in the basement membranes. In June testes it is absent (fig. 8).

## SEMINIFEROUS TUBULES

Figures 1 and 2 illustrate frozen sections of fall testes stained respectively with sudan III and sudan black B. Both coarse and fine lipid droplets occur within the seminiferous tubules. The coarse particles vary in size and are located irregularly at the periphery of the tubules near the basement membrane. The minute lipid particles are widely scattered, existing both at the periphery and toward the center of the tubules. In the latter region they are maximal in number and appear to be located within the cytoplasm of spermatids as well as within globoid cytoplasmic masses freed into the lumen of the tubules during cytomorphosis of spermatids into spermatozoa. In addition to these various lipid droplets, the heads of the spermatozoa appear to be faintly stained, especially when viewed in profile (fig. 2). Since the large sudanophil droplets near the basement membrane do not seem

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Explanation of Figures

All of the sections illustrated on this plate were counterstained with Mayer's paracarmine.

5. Section of testis of a Virginia deer (Oct. 19) showing the distribution of alkaline phosphatase. Fixed in chilled 80% alcohol. The section was incubated in fructose diphosphate at pH 9.4 for 6 hours. ×220.

6. The same, utilizing yeast nucleic acid as substrate and incubating at pH 9.4 for 6 hours. In this and the previous photographs observe the intense phosphatase reaction of the seminiferous tubules and the surrounding basement membranes. The interstitial tissue is quite faintly stained excepting the dark reaction of the walls of the arteries. The apparent nuclear staining in this and the preceding section is partly due to the red color imparted by the counterstain. ×220.

7. Section of a testis of a Japanese deer (Nov. 13) showing the pattern and intensity of acid phosphatase. Fixation in chilled acetone. Section incubated with sodium glycerophosphate at pH 4.7 for 48 hours. The reaction is confined mainly to the cells of the seminiferous tubules. ×140.

8. Section of a testis of a Japanese deer (June 13) incubated with sodium glycerophosphate at pH 4.7 for 48 hours. Observe the difference between the November testis (preceding photograph) and the June testis shown in this figure. In June the shrunken, inactive seminiferous tubules, as well as the interstitial tissue, are essentially negative for acid phosphatase. ×140.

9. Section of a testis of a Japanese deer (June 13) incubated with sodium glycerophosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. The relatively slight reaction for alkaline phosphatase is confined almost entirely to the contents of the shrunken tubules. Neither the basement membranes nor the blood vessel walls have reacted. Compare with the intense reactions for alkaline phosphatase in fall testes (figs. 5 and 6). ×140.

to be located in either spermatogonia or spermatocytes, they are probably situated in the cytoplasm of Sertoli cells. The lipid droplets are not birefringent, but the sperm tails and cell membranes are, a birefringence which is not abolished by extraction with acetone. In the testis of a November Virginia deer examined in ultraviolet light,

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PLATE 3

Explanation of Figures

10. A group of interstitial cells from the testis of a Virginia deer (Oct. 19). Formalin-fixed, frozen section stained with sudan black B for 7 minutes. Compare with figure 1. ×10 ocular; =40 objective.

11. Another section from the same testis stained with Schiff's reagent, showing the depth and intensity of the plasmal reaction. Compare with figure 4. Observe that the plasmal reaction coincides in its pattern with that of the lipid in the previous section. ×10 ocular; ×40 objective.

12. A group of interstitial cells from a June testis (Virginia deer). Formalin-fixed, frozen section stained with sudan black B for 7 minutes. Compare with figure 10, observing the greatly diminished quantity of stainable lipid in the June testis. The plasmal reaction of the June testis (not illustrated) was similarly diminished. ×10 ocular; ×90 objective.

13. The epithelium of the distal portion of the ductus epididymidis showing the distribution of alkaline phosphatase. Virginia deer on Oct. 19. The borders of the columnar cells and the stereocilia react strongly as does also the cytoplasm of the small basal cells. Fixation in chilled 80% alcohol. Section incubated with fructose diphosphate at pH 9.4 for 3 hours. Counterstained with paracarmine. Compare with figures 23 and 27. ×10 ocular; ×90 objective.

14. The epithelium of the proximal portion of the ductus epididymidis of the same animal showing the distribution of acid phosphatase. The nuclei of 2 migrating lymphocytes are visible in the epithelium. The terminal bars at the surface of the epithelial cells react intensely. The stereocilia are faintly stained, and to the right deeply stained spermatozoa are visible. Fixation in chilled acetone. Section incubated in sodium glycerophosphate at pH 4.5 for 24 hours. Counterstained with paracarmine. Compare with figure 30. ×10 ocular; ×40 objective.

15. Spermatids from a seminiferous tubule of a Japanese deer killed on November 13. Fixed in Rossman's fluid and stained by the periodic acid-Schiff method. The acrosomes and portions of the acroblast applied to the nucleus react positively—staining not prevented by saliva and hence not attributable to glycogen. ×7 ocular; ×90 objective.

16. Spermatozoa (from ductus epididymidis) stained by the periodic acid-Schiff technique following fixation in Rossman's fluid. Virginia deer on Oct. 19. ×10 ocular; ×90 objective.

17. Spermatozoa from the same deer, fixed in Zenker's fluid and stained by the Feulgen method revealing the amount and distribution of desoxyribonucleic acid. ×10 ocular; ×90 objective.

18. Spermatozoa of a Japanese deer (Nov. 13) fixed in Zenker's fluid and stained with eosin and methylene blue. ×15 ocular; × 90 objective.

19. Spermatozoa of a Virginia deer (Oct. 19) fixed in Rossman's fluid and stained with Masson's triacid mixture. ×15 ocular; ×90 objective.

20. Spermatozoa of the same deer fixed in 4% basic lead acetate and stained in a ½% aqueous solution of toluidin blue, illustrating the absence of any metachromatic staining. ×7 ocular; ×90 objective.

21. Spermatozoa in the ductus epididymidis of the same deer showing the alkaline phosphatase reaction after incubating a section with fructose diphosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. ×15 ocular; ×90 objective.

22. Spermatozoa in the ductus epididymidis of a Japanese deer in November stained for acid phosphatase. Section incubated with sodium glycerophosphate at pH 4.7 for 48 hours. Fixed in chilled acetone. ×15 ocular; ×90 objective.


Plate 3

a number of reddish-yellow granules and droplets were observed in the basal portion of the tubules. The yellow color, interpreted as being due to lipochrome, seemed to be associated with the coarser sudanophil droplets observed in the preparations stained with sudan dyes. A few similar reddish-yellow droplets were present in the interstitial tissue. The epithelial cells of the seminiferous tubules do not give a specific plasmal reaction (fig. 4), and the spermatozoa are quite unstained. In the inactive epithelium of the June tubules the lipid droplets are fewer in number and smaller in size.

In fall testes cytoplasmic basophilia is evident in the cells of the seminiferous tubules in eosin-methylene blue preparations. Basophilia is slight in the spermatogonia and spermatocytes, but increases in amount in the cytoplasm of the spermatids. In the globoid cytoplasmic residues which become detached from the spermatids it forms a delicate blue stippling on a faint lavender background. In June testes cytoplasmic basophilia is minimal.

In the tubules of fall testes the periodic acid-Schiff method brings out a variety of stained structures (fig. 3). The basement membranes are intensely stained, and within occasional tubules vivid red droplets are present in some of the Sertoli cells. Toward the centers of the tubules, staining of spermatids and of spermatozoa is observed (fig. 3, 15 and 16). In the spermatids red material is visible in hemispherical contact with the nuclear membrane in addition to a deeply stained red body which is either close to or in contact with the nucleus (fig. 15). The former is interpreted as being part of the acrosome and the latter as the acroblast. The heads of the spermatozoa appear in profile as red spindles and in full view as paler spatulate objects (figs. 3 and 16). In addition, pale stained material is seen in the tubular lumen, more especially in those segments of the tubules in which spermatozoa are differentiating. This stained substance is interpreted as being residual cytoplasm released from spermatids in the course of their cytomorphosis into spermatozoa.

Except for the dark red droplets present in some of the Sertoli cells, none of the elements mentioned above is affected by the use of saliva. Thus, only the former are regarded as being glycogen. Of the various periodic acid-Schiff positive elements described above, the Bauer-Feulgen technique, stains only the droplets of glycogen.

In the inactive June testes, some of the shrunken seminiferous tubules contain flocculent material in the tubular lumens, which is intensely colored by both the periodic acid-Schiff and Bauer-Feulgen methods. The solubility of this substance in saliva indicates that it is glycogen. The residual spermatogenic cells do not appear to be specifically stained.

During the fall the seminiferous tubules exhibit most intense reactions for alkaline phosphatase (figs. 5 and 6). Besides the basement membranes, the epithelial elements of the tubules stain deeply. The

reaction involves the cytoplasm of spermatogonia, spermatocytes and spermatids, being relatively faint in the spermatogonia and reaching extreme intensity in the spermatids. The nuclei also contain alkaline phosphatase, although the dark nuclear staining apparent in figures 5, 6 and 9 is attributable partly to the counterstain. The spermatozoa are also stained. In the sperm heads, seen in profile, it appears as though the enzyme were located in a cytoplasmic membrane enclosing the nucleus, because not infrequently a central nuclear area stained by paracarmine can be faintly recognized surrounded by a brown sheath. It seems also as though the reaction were present in the cytoplasm of the Sertoli cells between the germinal cells, especially in the regions where the spermatids and spermatozoa are attached. Finally, a reaction is present in the cytoplasmic material which is liberated into the lumen of the tubules during the cytomorphosis of the spermatids into spermatozoa. In the shrunken tubules of June testes the reaction for alkaline phosphatase is considerably reduced (fig. 9). The enzyme is present for the most part free in the lumens of the tubules and in the cytoplasm of the surrounding epithelial cells. There is none in the basement membranes. In several specimens the centers of the atrophic tubules contained somewhat more than figure 9 indicates.

Acid phosphatase is present in the tubules of fall testes, occurring both in the nuclei and cytoplasm of the cells (fig. 7). None is encountered in the basement membranes. Some is present in the cytoplasm of the spermatogonia, slightly more is visible in the spermatocytes and still more is present in the spermatids and spermatozoa. The rather intense staining of the nuclei is associated mainly with the chromosomes. There appears to be some also in the seemingly fibrillar, distal cytoplasm of the Sertoli cells, as well as a widespread, faint, punctage reaction in the lumens of the tubules. In June testes no acid phosphatase has been encountered (fig. 8).

## EPIDIDYMIS

Observations on the epididymis have been confined principally to

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Explanation of Figures

23. Ductus epididymidis of a Japanese deer in November stained for alkaline phosphatase. Section incubated in sodium glycerophosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. Figure 13 shows a detailed view of such a preparation. ×140.

24. The same from a deer killed in June, illustrating the complete absence of alkaline phosphatase. ×140.

25. Ductus epididymidis of a Japanese deer in November stained for acid phosphatase. Section incubated in sodium glycerophosphate at pH 4.7 for 48 hours. Fixation in chilled acetone. ×140.

26. The same from a deer killed in June, illustrating the complete absence of acid phosphatase. ×140.

27. Ductus epididymidis of a Virginia deer in October stained for alkaline phosphatase. Section incubated in fructose diphosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. ×190.

28. A ductus efferens from the same section as the preceding, illustrating the distribution of alkaline phosphatase. ×190.


PLATE 4

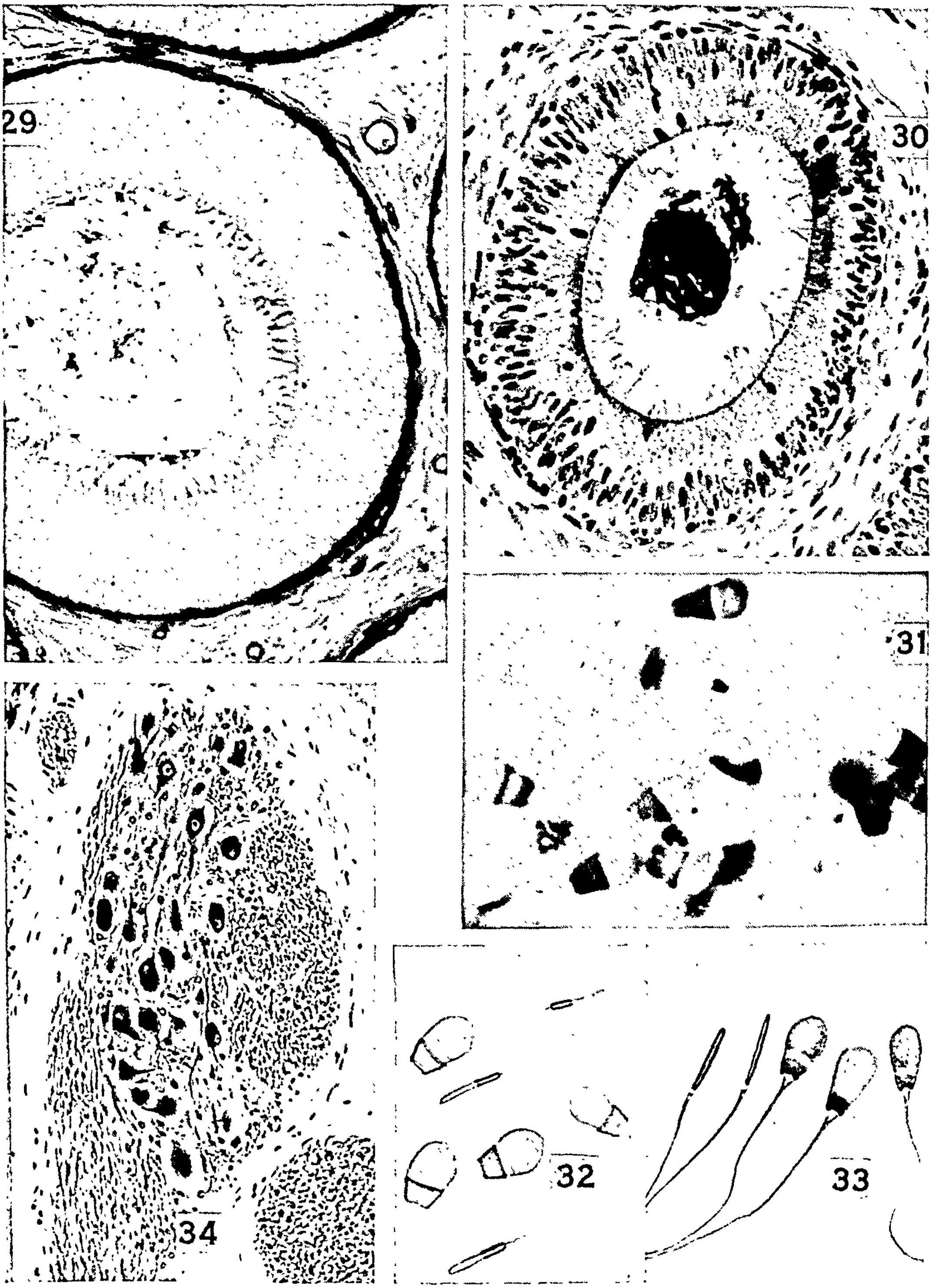


PLATE 5

the ductus epididymidis, but since a few of the sections have included some of the efferent ducts, these will be occasionally mentioned.

*Ductus epididymidis.* This duct appears to vary some in different parts of its length. We have not investigated these differences except to note that the epithelium of the proximal highly convoluted portion of the duct possesses taller cells bearing longer and more delicate stereocilia (figs. 14, 29 and 30) than the distal part where the cells and cilia are lower (figs. 13, 23, 25 and 27). In regard to histochemical features no differences have been noted in these 2 regions.

The epithelium of the ductus epididymides of the several deer examined contains relatively little stainable lipid. With sudan black B a few stained droplets are encountered in the small basal cells, but there are none in the columnar cells. No noticeable seasonal differences in regard to lipid have been observed.

In eosin methylene blue preparations after fixation in Zenker's fluid, the outer halves of the columnar cells including the border of stereocilia are strongly eosinophilic. In the deeper halves of the cells in the nuclear region a faint bluish lavender stippling is visible, indicating the presence there of some degree of cytoplasmic basophilia. The cytoplasm of the small basal cells is distinctly acidophilic.

In preparations fixed in basic lead acetate and stained with toluidin blue no metachromasia is visible in the various elements of the epididymis, excepting numerous mast cells in the stroma, which are strongly metachromatic.

The nuclei of the columnar epithelial cells are elongated and vesicular, whereas those of the basal cells are slightly shrunken and possess a relatively dark nuclear membrane. In addition to these 2 types,

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Explanation of Figures

29. Ductus epididymidis of a Virginia deer (Oct. 19). Fixed in Rossman's fluid and stained by the periodic acid-Schiff method. The basement membrane, the walls of the blood vessels and the mass of spermatozoa within the duct react strongly, but none of these reactions is prevented by exposure of the sections to saliva. Neither the epithelial cells nor the stereocilia are specifically stained. ×240.

30. Ductus epididymidis of a Japanese deer (Nov. 13) showing acid phosphatase in the nuclei, in the distal portions of the epithelial cells, in the stereocilia, and in the mass of spermatozoa within the lumen. The darkly stained nuclei of migrating lymphocytes are scattered throughout the epithelium. Fixed in chilled acetone. Section incubated in sodium glycerophosphate at pH 4.7 for 48 hours. Figure 14 shows a detailed view of this preparation. ×240.

31. Spermatozoa in the ductus epididymidis of a Virginia deer in October, showing their staining by Bodian's protargol method. Fixation in Rossman's fluid. ×1620.

32. Drawing of the same. Rossman's fixative and Bodian's protargol stain. ×15 ocular; ×90 objective.

33. Spermatozoa in ductus epididymidis stained with Heidenhain's iron-hematoxylin after fixation in Rossman's fluid. ×15 ocular; ×90 objective.

34. Sympathetic ganglion in capsule of seminal vesicle stained for acid phosphatase. Tissue fixed in chilled acetone and section incubated with sodium glycerophosphate at pH 4.7 for 48 hours. Counterstained with paracarmine. Observe the intense staining of the nerve cells and of the nerve fibers. ×160.

small, intensely chromatic nuclei are encountered scattered irregularly throughout the epithelium and present also in the tunica propria. With methylene blue or with Feulgen's stain these have the characteristics of the nuclei of lymphocytes. Some of them are surrounded by a clear halo of faintly stained cytoplasm. These cells are interpreted as being lymphocytes in process of migrating through the wall of the ductus epididymidis. Their nuclei contain acid phosphatase as illustrated in figures 14 and 30.

The long stereocilia, present on the cells in the proximal part of the epididymal duct, form bundles protruding into the lumen. The cilia do not possess basal bodies, but the surfaces of the columnar cells are equipped with terminal bars which produce a pattern of dots and lines indicating the cell boundaries. The terminal bars are readily seen after Zenker fixation and Masson's stain. In acid phosphatase preparations (figs. 14 and 30) the enzyme appears to be concentrated in or on the substance of the bars (figs. 14 and 30). Its appearance might be construed as related to basal bodies but analysis of the situation implicates terminal bars.

Seasonal differences in the cytology of the ductus epididymidis are slight, as revealed by eosin and methylene blue or Masson's stain. The major difference is the absence of spermatozoa in the epididymis of early June. Although the duct seems to be much narrower than in the active period, the epithelium is quite tall and is provided with normal appearing cilia. The nuclei of the basal cells seem to be more conspicuous than in the fall.

Stained by the Bauer-Feulgen method the ductus epididymidis is negative. After staining by the periodic acid-Schiff technique its appearance is illustrated in figure 29. The epithelial cells including the stereocilia are unstained. However, the basement membrane enclosing the duct and the mass of spermatozoa contained within the lumen are deeply colored. Since none of this staining is prevented by exposure to saliva, it is not due to glycogen.

In preparations of the epididymis from the fall months an intense alkaline phosphatase reaction is encountered in the stereocilia and in the cytoplasm of the small basal cells which form a discontinuous layer on the inner surface of the basement membrane (figs. 13, 23 and 27). The basement membrane and the well-defined muscle layer surrounding the duct react moderately (fig. 27). The nuclei do not react. The mass of spermatozoa contained in the duct lumen is very intensely colored (fig. 23). Alkaline phosphatase is also abundant in the efferent ducts (fig. 28). The reaction is intense in the basement membrane and in the underlying layer of muscle. On the free surface of the epithelium it occurs particularly on the crests of the scalloped folds. In June, when spermatogenesis is in abeyance, alkaline phosphatase is not encountered in the epididymis.

In the fall, acid phosphatase is also readily demonstrable (figs. 14, 25 and 30). Its distribution is different from that of alkaline phos-

phatase. It occurs principally in the cell nuclei of the epididymal duct and does not bring out either the basement membrane or the muscle layer. It occurs also in the distal halves of the epididymal epithelium increasing toward the surface and outlining the terminal bars with great sharpness (figs. 14 and 30). The stereocilia are delicately stained. Similarly to alkaline phosphatase, it is present in considerable concentration in the mass of spermatozoa in the lumen of the ductus epididymidis (figs. 25 and 30).

In June epididymides, on the contrary, it is practically absent, giving only a faint tinge to the cell nuclei (fig. 26).

## SPERMATOZOA

The methods used in the present study offer an opportunity to observe certain cytochemical properties of spermatozoa. Because the observations are varied and numerous, they are assembled here under a separate heading. Insofar as we are aware, spermatozoa have not been hitherto examined in reference to their staining by sudan black B, the periodic acid-Schiff method or Bodian's protargol method. The spermatozoa were available for examination in fixed sections of both testis and epididymis.

*Sudan black B.* With this dye the sperm heads appear to be faintly stained, indicating the presence of a lipid component (figs. 1 and 2) which, it seems reasonable to believe, may be contained in the sheath surrounding the head.

*Schiff's plasmal reaction.* By this method the spermatozoa remain unstained. Since this reagent, as we have used it, stains lipids of the interstitial tissue very distinctly (fig. 11), there is no apparent reason to doubt the validity of the negative finding for the spermatozoa. This qualifying statement is added because Marza ('31) has described a diffuse staining of spermatozoa in various mammals by the "plasmal or aldehyde reaction of Feulgen," a finding which we are unable to confirm.

*Glycogen.* Glycogen is not demonstrable in the spermatozoa of deer by either the Bauer-Feulgen or periodic acid-Schiff reactions. With the use of iodine, applied in 2 different ways, Marza ('31) claims to have found small quantities of glycogen in the middle piece of the spermatozoa of various mammals.

*Periodic acid-Schiff method.* This method colors the sperm heads quite distinctly (figs. 3 and 16), but does not stain the middle pieces or tails. The staining of the head of the spermatozoon is quite probably related to the staining of the acroblast and acrosome of the spermatids of the preceding stages (fig. 15). Since this staining of the germinal cells is not influenced by the use of saliva, it cannot be attributed to glycogen. Moreover, since none of these elements of the cells reacts metachromatically with toluidin blue, an acid mucopolysaccharide does not appear to be involved. A substance of unknown composition suggests itself, in which, upon oxidation with periodic acid, exposed

aldehyde radicles become stained by Schiff's reagent. Gersh ('48) has recently announced that the Golgi apparatus of certain cells contains a glycoprotein which gives a periodic acid-Schiff reaction. The material of the Golgi apparatus is known to become incorporated into the acroblasts and acrosomes (cf. Metz, '32), and it may be for that reason that these structures give a positive reaction. Since during subsequent cytomorphosis of the spermatids part of the material of the acroblast is cast off while other parts become associated with the sperm head, account would also be taken of the Schiff positive reactions of these 2 derivatives.

*Basophilia.* The condensed chromatin of the heads of the spermatozoa stains intensely with basic dyes (figs. 16 and 18) and by the Feulgen method (fig. 17). Basophilia is confined exclusively to the head of the spermatozoon (fig. 20) and coincides exactly, it appears, with the nuclear area of condensed chromatin which gives the specific Feulgen reaction for desoxyribonucleic acid. Accordingly, cytoplasmic basophilia attributable to ribonucleic acid appears to be lacking. Nor is there any metachromasia visible after fixation in basic lead acetate and staining with toluidin blue. Stained with Masson's mixture of acid dyes, the nucleus is faintly colored with orange G, and the middle piece and tail with light green (fig. 19). The middle piece and tail have a strong affinity for acid dyes, as is illustrated further by specimens stained with a mixture of eosin and methylene blue (fig. 18).

*Phosphatases.* The masses of spermatozoa within the lumen of the epididymis exhibit very intense reactions for both acid and alkaline phosphatases (figs. 23, 25 and 30). Examined under higher magnification the individual spermatozoa are much paler, but reveal nevertheless a distinct complement of both acid and alkaline phosphatases (figs. 21 and 22). The 2 enzymes appear to be present throughout the length of the spermatozoon but they differ in character in some slight respects. When prepared for alkaline phosphatase, the anterior half of the spermatozoon heads is stained more darkly than the posterior portion, whereas with acid phosphatase the reverse is true. Moreover, in the acid phosphatase preparations there seems to be a gap in the region of the neck piece where enzyme is lacking, whereas with alkaline phosphatase (fig. 22) the necks are stained. It is difficult to decide whether these enzymes are localized principally in the interior of the spermatozoa or mainly on their surfaces. Since alkaline phosphatase appears to be associated with the surface of the cells of both ductus epididymidis and ductuli efferentes (figs. 27 and 28), it is possible that the phosphatase associated with the spermatozoa may also be located on or in the surface layer. Indeed, it is sometimes evident, in spermatozoa seen in profile, that the paracarmine-stained nucleus of the head is ensheathed by a layer containing alkaline phosphatase. On the other hand, since acid phosphatase appears definitely to be a component of the nuclei of the antecedent spermatids, it seems probable that it is also located inside the nucleus of the spermatozoa.

*Bodian's protargol method.* By this technique the heads of the spermatozoa are very sharply stained (figs. 31 and 32). The acrosomic cap (galea capitis), which has the appearance of a thin, structureless membrane covering the anterior two-thirds of the head, is grey. Its posterior border forms a faint curved line across the head. The posterior nuclear cap covering the caudal third of the head is intensely impregnated with silver. Between the edges of the anterior and posterior caps a narrow, unstained, lens-shaped field is visible through which the eye detects the transparent interior of the head. Spermatozoa viewed in profile illustrate further the intensely black posterior cap, the faintly stained anterior cap and the unstained intervening zone (fig. 32) Posteriorly, the head terminates in a concave edge which coincides exactly with the border revealed by the Feulgen method or toluidin blue (cf. figs. 17 and 20). Occasionally the body and tail of a spermatozoon are just barely perceptible.

*Heidenhain's iron hematoxylin.* By this stain all parts of the spermatozoon excepting the neck are brought out (fig. 33). It reveals the topography of the sperm with much the same clarity as with eosin-methylene blue or Masson's triple stain (figs. 18 and 19). By all three of these methods a pyramidal-shaped, unstained area of the neck piece is visible just caudad to the concave posterior border of the head.

## SEMINAL VESICLES

The accessory male sex glands of deer have not been identified or investigated in the present study, except for a pair of glandular organs situated near the base of the bladder. From their location and histology, we regard these structures as being seminal vesicles. Each consists of a firm, tortuous, glandular mass compactly ensheathed by connective tissue. Each varies from 2 cm. in length, in the sexually inactive period, up to 3 or 4 cm. in the fall, and has a diameter of about 1 cm.

The seminal vesicles undergo pronounced seasonal histological changes (Wislocki, '43). In June they present numerous small alveoli lined by relatively low cells. At this time the connective tissue stroma between the shrunken alveoli is relatively abundant. In the lumens of the acini, in this month, there is a small amount of seemingly inspissated secretion consisting principally of an amorphous, brownish-black substance. By October the gland is very much altered: the lumina are much enlarged and are filled with a copious globular and granular secretion which has replaced the scanty, brown secretion of the inactive period. The epithelial cells have become exceedingly tall. from an average of 18 to 20 $\mu$ in June having attained a height of about 40 $\mu$. With the great enlargement of the acini the stroma has become stretched and thinned out and has assumed a more complex branching pattern. To the general histological features noted on a previous occasion (Wislocki, '43), the present study adds a few additional observations derived from histochemical methods.

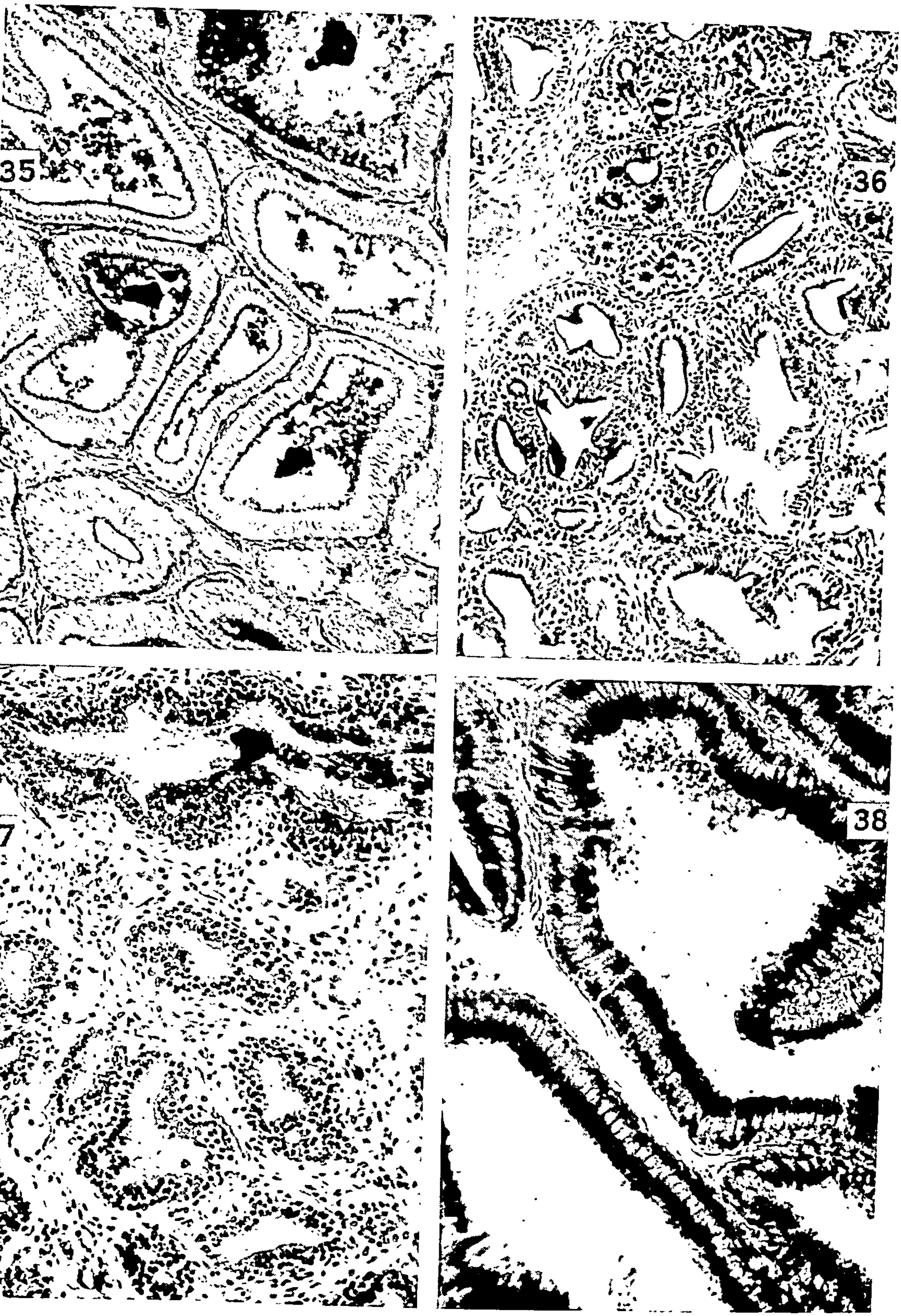


PLATE 6

Lipids are abundant in the epithelium of the fall seminal vesicles. In the Virginia deer the character of the lipid material is illustrated in figure 38. Minute sudanophilic particles are present in extreme amounts in the supranuclear and infranuclear regions of the cells. In a Japanese deer from the same period the character of the fat differs somewhat from that seen in the Virginia deer. Instead of a multitude of minute particles, the lipid occurs in the main in somewhat larger droplets located principally in the infranuclear region. In June testes sudanophil material is also present in the characteristic pattern for the species but in lesser quantities.

Cytoplasmic basophilia is not a feature of the epithelium of the seminal vesicles. Instead, the cytoplasm of the cells has a strong affinity for acid dyes.

After fixation in basic lead acetate and staining with toluidin blue, no metachromasia is visible, except for mast cells which are extremely numerous in the stroma. The granules of these cells are stained a deep purple.

There is some glycogen in the seminal vesicles, demonstrable by the Bauer-Feulgen and periodic acid-Schiff methods as checked by saliva controls. In specimens from the fall months it is present in the secretion in the lumens of some of the acini as well as also in the cytoplasm of some of the epithelial cells. In June specimens none is found free in the alveoli, but some is present in the cytoplasm of many of the epithelial cells.

Acid and alkaline phsophatases occur in the seminal vesicles. In the October gland alkaline phosphatase occurs abundantly at the distal margins of the epithelial cells, in the secretion filling the lumens and in the interacinar stroma (fig. 35). The apparent staining of the epithelial nuclei in the photograph is due to the use of paracarmine as a counterstain and not to phosphatase. In June much less alkaline

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Explanation of Figures

35. Seminal vesicle of Virginia deer in October showing the reaction of alkaline phosphatase. Tissue fixed in chilled 80% alcohol and section incubated with sodium glycerophosphate at pH 9.4 for 6 hours. Counterstained with paracarmine. Observe the intense phosphatase reaction in the secretion, at the distal borders of the cells and in the stroma. The apparent staining of cell nuclei is due solely to the counterstain. ×140.

36. Seminal vesicles of Virginia deer in June for comparison with the preceding showing the reaction of alkaline phosphatase. Section prepared by the same technique as the preceding one. Observe in the June testes that the acini are smaller and the epithelium lower, and that there is little secretion in the lumens. The phosphatase reaction is limited to the distal ends of the epithelial cells. The nuclear reaction seen in this section is due solely to the counterstain. ×140.

37. Seminal vesicle of a Japanese deer in November stained for acid phosphatase. Tissue fixed in chilled acetone and section incubated in sodium glycerophosphate at pH 4.7 for 48 hours. Counterstained with paracarmine. The reaction of acid phosphatase, in contrast to that of alkaline phosphatase (fig. 35), is located principally in the nuclei. The nuclei are an intense brown or tan in the original preparation. ×140.

38. Seminal vesicle of Virginia deer in October. Frozen section of a formalin-fixed gland stained for 7 minutes with sudan black B. Finely divided sudanophil material is abundantly present in the epithelium in both supra- and infranuclear positions. ×220.

phosphatase is present (fig. 36). The shrunken gland lumens are for the most part empty, except for scattered masses of inspissated brownish secretion. A faint alkaline phosphatase reaction occurs in the distal cytoplasm of some of the epithelial cells and this becomes more intense along the free surfaces of the cells in some alveoli. In this photograph, too, the dark coloration of the nuclei is attributable to counterstaining with paracarmine. It is apparent that the October gland is richer in alkaline phosphatase than the June one.

Acid phosphatase is also present in the seminal vesicles, but, contrary to alkaline phosphatase, it is localized almost entirely in the nuclei of the epithelial cells (fig. 37). The darkly stained nuclei in the photograph are intensely brown or tan in the original preparation, in contradistinction to the nuclei of figures 35 and 36 which are red or pink in the original sections due to staining with paracarmine. Besides the acid phosphatase in the nuclei of the epithelium, there is a lesser amount of enzyme in the nuclei of the stroma. In the seminal vesicles of a June deer the acid phosphatase reaction is very much fainter.

In one of the fall specimens several sympathetic ganglia and a number of nerve bundles were encountered in the capsule of the seminal vesicles. The ganglion cells and the nerve fibers show intense reactions for acid phosphatase (fig. 34).

## SUMMARY AND CONCLUSIONS

The testes, epididymides and seminal vesicles of deer undergo seasonal changes. In the present investigation these organs are compared in their active and inactive states with reference to lipids, steroid hormones, glycogen and acid and alkaline phosphatases.

The cells of the interstitial tissue in the fall contain lipid droplets which are sudanophil and birefringent, give a positive plasmal reaction, exhibit yellow fluorescence and are soluble in acetone. The combination of these reactions indicates that steroid hormones are formed in the interstitial tissue. In June testes these reactions are much less intense.

The interstitial cells contain traces of alkaline phosphatase and a little cytoplasmic basophilia, but no glycogen. The walls of the arterioles and the basement membranes surrounding the seminiferous tubules are rich in alkaline phosphatase but this disappears in the inactive period.

The cells of the seminiferous tubules of the active fall testes contain large amounts of both acid and alkaline phosphatases. Alkaline phosphatase is present in the cytoplasm of the germinal cells, being particularly intense in the spermatids. The nuclei are less deeply stained. Acid phosphatase occurs in both nuclei and cytoplasm of the germinal cells, the cytoplasmic reaction reaching maximum intensity in the spermatids. Both acid and alkaline phosphatases appear to be present in the cytoplasm of the Sertoli cells, and they occur also in the cytoplasm cast off from the spermatids into the lumen of the

tubules. In June these enzymes are much decreased.

Glycogen occurs in some of the Sertoli cells in active testes, while in inactive ones it is present in many of the tubular lumens. The acroblasts and acrosomes of the spermatids, as well as the heads of the spermatozoa, are stained red by the periodic acid-Schiff method, a reaction not prevented by the use of saliva and indicative of a glycoprotein.

The cytoplasm which is cast off by the spermatids contains lipid droplets, basophilic substance, alkaline and acid phosphatases, and a glycoprotein which reacts with the periodic acid-Schiff reagents.

The cells lining the ductus epididymidis contain both acid and alkaline phosphatases. The former is localized principally in the nucleus, distal cytoplasm and ciliated border of the columnar cells. The enzyme delineates the terminal bars at the surfaces of the cells. Alkaline phosphatase is localized in the ciliated border of the columnar cells and in the cytoplasm of the small basal cells. Lymphocytes, whose nuclei contain acid phosphatase, are seen migrating through the epithelium of the ductus epididymidis.

The spermatozoa exhibit various reactions. Their heads, besides being stained by the periodic acid-Schiff technique, are faintly stained by sudan black B. Both acid and alkaline phosphatases occur in the spermatozoa. The latter seems to be present in the cytoplasmic sheath covering the head. By Bodian's protargol method the heads are very sharply delineated: the anterior head cap stains grey and the posterior cap black, with a narrow intervening zone which is unstained.

Seasonal changes, in reference to size and secretory activity, are noticeable in the seminal vesicles. The seasonal distribution of acid and alkaline phosphatases, lipids and glycogen in these glands is briefly described.

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# EARLY EFFECTS OF TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES OF CASTRATE RATS[1,2]

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THE LONG term effects of administered testosterone to castrate animals are well known. The synthesis of protein, the rise in acid phosphatase of the prostate gland (Huggins, et al. 1939), and the marked production of fructose in the seminal vesicles (Mann 1946a, 1946b) are well-recognized biochemical changes. Some histological studies have been carried out after the androgens have acted for short periods of time. Tislowitz (1939), using colchicine, demonstrated that profuse mitoses in the seminal vesicles of castrate mice occurred about 24 hours after the injection of testosterone. Burkhart (1939, 1942) has shown that testosterone propionate initiates hypertrophy of the cells in the prostate of the castrate rat 23 hours after injection, and cell division in 36 hours. The relation of these morphological changes to the biochemical phenomena, however, has not been explored. Experiments were therefore designed to determine at what time alterations in certain biochemical reactions appeared after injection of testosterone propionate.

## METHODS

Male rats from Sprague-Dawley, Inc. were castrated when 30 to 33 days old. The preparation described by Hays and Mathieson (1945) was used, since they demonstrated that the seminal vesicles of these castrate rats exhibited the same increase in weight when the animals were injected with 1 mg. of testosterone propionate at any time from 7 to 350 days after castration. The treated rats were injected subcutaneously with a single dose of 1 mg. of testosterone propionate in 0.2 ml. of sesame oil. The seminal vesicles were weighed on a Roller-Smith microbalance immediately after their removal from the animal. The water content of the glands was determined by drying the tissues to a constant weight at 85°C. to 90°C., the barometric pressure usually being about 630 mm. mercury. The chloride content was determined by the micro-diffusion method described by Clark, Levitan, Gleason, and Greenberg (1942). The chloride which diffuesd into the potas-

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sium hydroxide was then determined by the iodometric method of Van Slyke and Hiller (1947). The sodium content of the glands was determined by the method of Leva (1940). This procedure was carried out in the cold room (temperature about +2°C.). The oxygen consumption was measured in the Warburg apparatus at 37°C. The tissues were kept in ice-cold buffer solution from the moment of weighing the glands. The glands were homogenized and the protein precipitated with trichloracetic acid. Aliquots of the protein-free filtrate were used for the fructose determinations. The fructose content of the seminal vesicles was determined by the method of Reinecke (1942).

## RESULTS

Greene and Burrill (1940) noted an increase in the weight of the seminal vesicles 24 hours after injection. A maximum response to a dose of 1 mg. occurs 96 hours after injection (Hays and Mathieson, 1945). The data in Table 1 indicate that the increase in weight of the seminal vesicles of castrate rats 10 hours after the injection of testosterone propionate is significant as also are those 15 and 20 hours after injection. The calculation of an "overall t value" for the 5 groups of animals 10 hours after injection gives a value of 8.46, which is very highly significant.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON THE WEIGHT OF THE SEMINAL VESICLES OF THE CASTRATE RAT

| Hours after injection | No. of rats | Average weight of glands with S. E. | Average weight of glands from control rats with S. E. | Difference of average weights | t | P |
|---|---|---|---|---|---|---|
| | | mg. | mg. | mg. | | |
| 5 | 5 | 6.70 ±0.30 | 6.33 ±0.25 | 0.37 | 0.91 | 0.4 |
| 10 | 30 | 8.67 ±0.21 | 7.33 ±0.15 | 1.30 | 4.84 | <0.01 |
| 10 | 16 | 7.17 ±0.25 | 6.44 ±0.22 | 0.73 | 2.18 | 0.05 |
| 10 | 21 | 8.04 ±0.18 | 6.99 ±0.17 | 1.05 | 4.20 | <0.01 |
| 10 | 24 | 7.66 ±0.17 | 6.65 ±0.17 | 1.01 | 4.31 | <0.01 |
| 10 | 21 | 7.45 ±0.17 | 6.69 ±0.17 | 0.76 | 3.20 | <0.01 |
| 15 | 5 | 7.66 ±0.35 | 6.33 ±0.25 | 1.33 | 3.18 | 0.02 |
| 20 | 36 | 9.53 ±0.22 | 6.63 ±0.17 | 2.90 | 10.53 | <0.01 |

The data summarized in Table 2 show that the water content of the seminal vesicles is significantly increased 10 hours after the injection of the hormone. There is a small, insignificant decrease in chloride concentration. The water content 20 hours after injection is greater and is accompanied by a significantly decreased concentration of chloride. There is also a decreased sodium concentration 20 hours after the administration of testosterone propionate.

Table 3 gives the distribution of the water in the seminal vesicles of castrate rats before and after the administration of the androgen, based on the analytical data in Table 2. The increase in the "chloride space" at the 10-hour period was 14%. The "non-chloride space" increased 26% during the same period. At the 20-hour period the increases were 22% and 97%, respectively. The increases in "sodium

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE ON THE WATER CONTENT, CHLORIDE AND SODIUM OF THE SEMINAL VESICLES

| | No. of groups of four rats each | Control<br>Average with S.E. | Ten hours after injection<br>Average with S.E. | Twenty hours after injection<br>Average with S.E. | t | P |
|---|---|---|---|---|---|---|
| % water | 6 | 80.8 ±0.15 | 82.1 ±0.18 | | 5.49 | <0.01 |
| % water | 7 | 79.7 ±0.64 | | 82.0 ±0.44 | 2.96 | 0.02 |
| Chloride, meq./l. | 6 | 86.9 ±1.41 | 85.6 ±1.09 | | 0.74 | 0.5 |
| tissue water | 2 | 91.4 ±3.10 | | 80.9 ±0.45 | 4.22 | 0.05 |
| | 3 | 95.1 ±2.52 | | 82.8 ±1.26 | 4.31 | 0.02 |
| Sodium meq./l. | 2 | 135.5 ±1.5 | | 115.0 ±3.2 | 5.35 | 0.02 |
| tissue water | | | | (3 groups of rats) | | |

space" and "non-sodium space" at the 20-hour period were 17% and 142%, respectively, With these calculations of "chloride space" and "sodium space" it is evident that the gland during the first 20 hours of growth incorporates most of the increased water into the cell contents.

With the increase in cell volume there is an accompanying increase in the organic constituents. In Table 3 it can be seen that the dry weights of the glands increased 6.4% during the first 10 hours and 22% during the first 20 hours. While there was a small increase in the amount of inorganic ions, these would not account for more than 10% of the change; the greater part of the gain must have been in organic compounds.

The data obtained from a number of experiments on the oxygen uptake of the seminal vesicles of castrate rats before and after treatment with testosterone propionate are shown in Table 4. When the oxygen consumption was measured with no added substrate the $Q_{O_2}$ of the seminal vesicles from the animals treated with the hormone for ten hours was 5.37 and the $Q_{O_2}$ of the glands of the untreated castrate controls was 4.21. The difference was statistically significant. The $Q_{O_2}$ for the seminal vesicles 20 hours after the injection of testosterone propionate and for slices of seminal vesicles from normal mature rats did not differ significantly from that found 10 hours after injection. The

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON THE DISTRIBUTION OF WATER IN THE SEMINAL VESICLES

| | Weight of Seminal Vesicles[1] | Dry weight | Water | "Chloride space"[2] | "Non-chloride space" | "Sodium space"[3] | "Non-sodium space" |
|---|---|---|---|---|---|---|---|
| | mg. | mg. | mg. | mg. | mg. | | |
| Control | 26.59 | 5.13 | 21.46 | 16.1 | 5.4 | | |
| Inj. 10 hours | 30.63 | 5.46 | 25.17 | 18.4 | 6.8 | | |
| Control | 27.61 | 5.16 | 22.45 | 17.6 | 4.8 | 18.9 | 3.6 |
| Inj. 20 hours | 37.17 | 6.30 | 30.87 | 21.4 | 9.5 | 22.2 | 8.7 |

[1] Total weight of the glands from four animals.
[2] Calculated from chloride values in Table 2, assuming all of the chloride is extracellular.
[3] Calculated from sodium values in Table 2, assuming all of the sodium is extracellular.

metabolic state of the gland is increased soon after the adminsitration of the hormone, and this level apparently persists as long as the hormone is present.

TABLE 4. EFFECT OF TESTOSTERONE ON THE $Q_{O_2}$ OF THE SEMINAL VESICLES

| No. of determinations | Substrate | Hours after injection | $Q_{O_2}$* | $Q_{O_2}$* of controls | Difference | t | P |
|---|---|---|---|---|---|---|---|
| 2 | glucose | 5 | 3.0 | 3.2 | −0.2 | | |
| 23 | none | 10 | 5.37 ±0.23 | 4.21 ±0.23 | 1.16 | 3.52 | <0.01 |
| 7 | glucose | 10 | 5.58 ±0.54 | 3.85 ±0.38 | 1.73 | 2.60 | 0.02 |
| 10 | sodium succinate | 10 | 6.79 ±0.18 | 5.51 ±0.26 | 1.28 | 3.78 | <0.01 |
| 7 | glucose +sodium succinate | 10 | 6.59 ±0.46 | 5.65 ±0.85 | 0.94 | 0.97 | 0.4 |
| 4 | glucose | 20 | 5.24 ±0.18 | 3.01 ±0.28 | 2.23 | 6.76 | <0.01 |
| 4 | glucose | slices of gland from mature rat | 5.66 ±0.45 | 3.00 ±0.46 | 2.66 | 4.07 | 0.02 |

* c. mm. $O_2$ per mg. dry weight per hour.

When glucose was added as substrate there was no appreciable change in the $Q_{O_2}$ of either the treated or the untreated groups; the difference between the two groups was again significant.

When sodium succinate was added, however, the oxygen uptake of each group showed an increase of similar order; the $Q_{O_2}$ of the seminal vesicles of the injected group remained significantly higher than that of the controls. When both glucose and sodium succinate were added the results were similar to those with succinate alone.

It was found that the addition of sodium fumarate did not increase the $Q_{O_2}$ of either the treated or untreated glands. This unexpected result was followed by experiments on the inhibition of oxygen uptake by sodium malonate. The addition of sodium malonate caused a 43% decrease in the $Q_{O_2}$ of the control glands, and a 37% decrease in the $Q_{O_2}$ of the treated glands (Table 5). The addition of 0.01M sodium fumarate to the system inhibited by the malonate did not affect the oxygen uptake.

In two experiments on anaerobic glycolysis it was found that the amount of acid produced was similar for the seminal vesicles from the treated and the untreated animals. The $Q_{CO_2}^{N_2}$ for the glands from the animals injected 10 hours before sacrificing was 7.5 and the $Q_{CO_2}^{N_2}$ of the

TABLE 5. EFFECT OF SODIUM MALONATE ON THE $Q_{O_2}$ OF THE TREATED AND UNTREATED SEMINAL VESICLES

Effect of the addition of sodium fumarate to the malonate-inhibited system

| | $Q_{O_2}$ | $Q_{O_2}$ with malonate | $Q_{O_2}$ with malonate +fumarate |
|---|---|---|---|
| Control | 4.2 | 2.4 | 2.7 |
| Injected | 6.0 | 4.0 | 3.7 |
| | 6.3 | 3.8 | 3.5 |

untreated controls was 7.0. These results indicate that the hormone does not alter the anaerobic glycolysis of the seminal vesicles to any marked degree.

In Table 6 the data show that the level of fructose in the seminal vesicles of the untreated castrate rats was lower than the level found 10 or 20 hours after the injection of testosterone propionate. In order to ascertain whether or not the increased fructose preceded the increased oxygen uptake, determinations were made at 5 hours and 8 hours. It was found that approximately the same level existed at these times as in the untreated gland. The increased fructose level is, therefore, found at the same time that the increased oxygen consumption is obtained. This would indicate a possible relation between the two processes.

TABLE 6. EFFECT OF TESTOSTERONE PROPIONATE ON THE FRUCTOSE CONTENT OF THE SEMINAL VESICLES

| | No. of determinations | Fructose mg./100 gm. tissue |
|---|---|---|
| Control | 4 | 9.1 |
| Injected 5 hours | 2 | 7.3 |
| Injected 8 hours | 2 | 9.9 |
| Injected 10 hours | 3 | 15.2 |
| Injected 20 hours | 1 | 15.7 |

## DISCUSSION

From the results of these experiments it appears that the primary effect of testosterone is on the intracellular metabolism of the seminal vesicles. A definite shift in the metabolic pattern, demonstrated by an increased oxygen consumption per unit weight of tissue, occurs as early as any measurable change in weight or shift in water and electrolytes. Once this has occurred, the new tissue which is formed also has this metabolic pattern since the oxygen consumption per unit weight of gland remains about the same even in the gland of the mature male.

The change in fructose concentration in the gland paralleled the change in oxygen consumption. In the uninjected animal and at 5 and 8 hours after injection the concentration was similar to the upper range of normal plasma concentrations. It then rose abruptly at 10 hours to a level which was markedly above those reported for plasma; this was maintained at 20 hours after injection. Similar values are given by Mann (1946b) for the mature rat prostate and seminal vesicle. Since the pattern of change of oxygen consumption and fructose concentration with time parallel each other it is probable that the two are associated phenomena.

The increase in intracellular water is probably the result of the accumulation of smaller, poorly diffusible molecules in the cell under the influence of the metabolic change. Fructose is one such molecule; it apparently does not passively diffuse from the seminal cells since the levels are markedly higher than the circulating fluids. This is par-

ticularly marked in the bull and ram where levels are 5 to 10 times the total reducing sugar level of the plasma. Even in man the levels in seminal plasma may be above 200 mg. per 100 cc. (Mann 1946b). Other molecules may also contribute to the osmotic change.

The effects of testosterone on the seminal vesicles contrast with those of another steroid, estradiol, on the uterus. The weight gain of the uterus after the injection of estrogen is not continuous. According to Astwood (1938), 6 hours after estrogen administration the weight of the uterus reached a first maximum followed by a decrease in weight between the sixth and twelfth hours, with a subsequent gain to the maximum response at 30 hours. The weight gain of the uterus at 6 hours was due almost entirely to an increase in water. Talbot, Lowry, and Astwood (1940) calculated from the electrolyte content of the uterus before injection, at 6 hours, and at 30 hours, that 80% of the fluid increase at 6 hours was extracellular, and that the fluid pattern of the unstimulated uterus again existed at 30 hours, the time of maximum weight gain. Hechter, Krohn and Harris (1941, 1942) reported a change in the permeability of the uterine capillaries soon after the injection of an estrogen.

At 6 to 12 hours after the injection of estrogen Kerly (1937) reported a slight increase in the anaerobic glycolysis of the uterus with a greater increase at 24 hours. The oxygen uptake of the uterus was significantly greater 36 hours after estrogenic stimulation (Kerly 1940). Carroll (1942) found a significant increase at 24 hours with a further increase at 45 hours. Apparently the first effect of estrogens is to change the fluid environment of the cell, after which the metabolic activity induced by these changes restores the normal distribution of fluid and electrolytes. This is in contrast to the androgen here studied, which appeared to act directly on the organic metabolism of the glandular cells; this, in turn, led to shifts in cells, water and electrolytes.

The first effect, histologically, of testosterone propionate on the seminal vesicles of the castrate rat that has been reported is hypertrophy (Burkhart 1942). An enlargement of the cells of the glands was noted 23 hours after the administration of the hormone, while increased mitotic activity could not be demonstrated until the 31 to 35-hour interval. Hays and Mathieson (1945) have shown that the maximum weight response to a single dose of 1 mg. of testosterone propionate occurred at 96 hours. The changes which we have observed are those, then, of the hypertrophic period.

While these studies did not involve the period of most rapid mitosis there is good reason to think that the multiplication of cells is a sequel of the primary metabolic shift, and does not involve marked new changes. As already mentioned, the oxygen consumption and fructose production of the mature gland do not differ markedly from those found 10 hours after injection. The increase in organic matter during the early period also indicates that synthesis is already accelerated.

The effects of testosterone on the metabolic pattern of the seminal

vesicles and prostate appear to be similar. Barron and Huggins (1944) found that the anaerobic glycolysis of the prostate of the castrate dog was the same as that of the prostate of the normal dog and of the gland of the castrate dog that had received testosterone propionate for 30 days. They concluded that testosterone regulates the oxidative phase of carbohydrate metabolism since the $Q_{O_2}$ of the prostates of normal dogs and of injected castrate dogs was higher than the $Q_{O_2}$ of the glands of the untreated castrate dogs. Similarly the change in the seminal vesicles appears oxidative, the anaerobic metabolism remaining relatively constant.

## SUMMARY

Ten hours after the injection of testosterone propionate into castrate rats the $Q_{O_2}$, weight, intracellular water, and fructose content of the seminal vesicles were increased. The $Q_{O_2}$ of the glands 20 hours after injection and the $Q_{O_2}$ of normal mature glands were similar to the $Q_{O_2}$ at 10 hours. The fructose content also rose between the fifth and tenth hours; thereafter this concentration was maintained. The metabolic effects seem primary. The continued growth of the seminal vesicles and the further increase of the intracellular volume were probably secondary effects of the hormone since the increased oxygen uptake could furnish the energy necessary for synthesis and the alteration of fluid distribution probably was the result of increased intracellular osmotic pressure.

The anaerobic metabolism of the seminal vesicles was unchanged after the injection of testosterone propionate. Succinate as substrate increased the $Q_{O_2}$ while the addition of glucose or fumarate had no effect.

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# NOTES AND COMMENTS

## HISTOCHEMICAL CHANGES IN THE ADRENAL CORTEX OF THE RAT IN ALLOXAN DIABETES

BENNETT and Koneff ('46) have shown recently that the adrenal cortex enlarges and contains increased quantities of lipid in rats made diabetic by injections of alloxan. Various considerations led these investigators to conclude that the adrenal changes were caused by the diabetic state and could not be ascribed to the toxic effects of alloxan.

A number of histochemical reactions for lipid substances are now available. Among these are several procedures which offer presumptive evidence for the presence of ketosteroids, since positive reactions are obtained in all of the endocrine glands which produce steroid hormones (cf. Demspey and Wislocki, '46), and since changes in the intensity of these reactions accompany altered physiological activity of the adrenal cortex (Deane and Greep, '46). The increased lipid in the adrenal cortex observed by Bennett and Koneff might represent a change in the steroid content of the gland. Accordingly, it was decided to apply the group of histochemical reactions to the adrenal glands of rats made diabetic by alloxan in an attempt to determine whether or not the increased lipid reflects a similar change in steroids.

### MATERIAL AND METHODS

Adult rats of the Long-Evans strain, weighing 130 to 150 grams, were used. In addition, one experiment was done on rats of the Sprague-Dawley strain. The diet consisted of Purina fox chow. All animals received intraperitoneal injections of alloxan (200 mg./kg.) on each of two successive days. Blood sugar determinations by the method of Folin and Svedberg ('30) were made at autopsy. The blood sugar values from all diabetic animals ranged between 275 and 620 mg. per cent.

Paralleling the experiments of Bennett and Koneff, one group of rats, consisting of diabetic animals and their uninjected controls, was killed 72 hours after the first injection of alloxan. A second group was killed 3 weeks later. At autopsy, the adrenal glands were weighed and fixed in 10% formalin. The glands were washed for several hours in running water and frozen sections were cut at 15 $\mu$. One section was mounted unstained for study of fluorescence and birefringence and others were stained with sudan IV and with the Schiff reagents. Similar sections were extracted with acetone and subjected to the same procedures.

### RESULTS

The adrenals of the diabetic rats were consistently larger than those from the corresponding controls. The increase in size was similar to that found by Bennett and Koneff. It was reflected histologically by a thickening of the zona fasciculata. The zona glomerulosa remained essentially unchanged.

At 72 hours, the zona fasciculata of the Long-Evans rats exhibited an

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increased sudanophilia throughout its entire extent. The fat-free transitional zone between the glomerulosa and fasciculata was wholly or partially obliterated. Increased numbers of finely divided birefringent particles were observed in the fasciculata. These deposits were especially heavy in the outer half of the layer, but were present in the cells bordering upon the reticularis as well, although in lesser concentrations. The Schiff reaction was also more intense and extended deeper into the cortex than did that observed in the adrenal glands of control animals. Two of the glands from this group exhibited an intensified and broadened zone of green fluorescence in the fasciculata, but the remaining two were indistinguishable from the normal control glands.

Three weeks after the injection of alloxan, similar but less marked changes were visible. Two of the 7 experimental animals exhibited an increased sudanophilia of the adrenal cortex. Increased numbers of birefringent particles were seen in 5 of these animals. The Schiff reaction was intensified in 6. Four of the 7 adrenals exhibited more intense and broader zones of fluorescence in the fasciculata than did the control glands.

An almost exactly opposite result was obtained in the group of rats of the Sprague-Dawley strain. This group, also killed 3 weeks after the injection of alloxan, exhibited marked reduction in the lipids of the fasciculata as judged by all of the reactions.

In general, the results show a good correspondence among the various histochemical tests as well as between the blood sugar level and the magnitude of change in the appearance of the adrenal. The sudanophilia, however, seems to be more variable than are the results from the other methods. Several examples exist in which little alteration in sudanophilia occurred in adrenals exhibiting marked changes in birefringence, fluorescence and in the Schiff reaction. This result is in keeping with other observations on the adrenal gland (Bennett, '40) and ovary (Dempsey and Bassett, '43) in which under various conditions the amount of lipid present does not parallel the amount of material exhibiting steroid reactions.

## DISCUSSION

The data presented above indicate that the lipid, present in increased quantities in the adrenal glands of alloxan-diabetic rats, exhibits enhanced reactions for ·ketosteroids. The adrenal cortex is now known to elaborate many specific steroid substances, and the present methods provide no means of distinguishing which particular compounds are involved. However, one clue exists which indicates that the carbohydrate-regulating, rather than the salt-conserving, principle of the adrenal is the one affected. This clue comes from the work of Deane and Greep ('46) who have shown that experimental situations causing a modified output of the carbohydrate-regulating hormones are associated with histological and histochemical changes in the zona fasciculata. Conversely, alteration of the electrolyte balance causes changes confined to the glomerulosa (Deane, Shaw and Greep, '48). These experiments lead to the thought that the carbohydrate- and salt-regulating hormones of the rat's adrenal are separately produced in the fasciculata and glomerulosa, respectively. Since the changes described above were confined to the fasciculata, it seems likely that the carbohydrate-regulating hormones of the corticosterone type were involved.

The difference in lipid content of the adrenals from the two strains of rats is of interest. The stimulus ordinarily causing increased lipid in Long-Evans rats invariably led to lipid depletion in Sprague-Dawley animals. Deane, Shaw and Greep ('48) have discussed the effect of stress on the adrenal gland, and have suggested that during mild stress the lipid and ketosteroid content of the cells increases, whereas in severe stress the discharge of droplets exceeds their rate of formation and the cellular content is diminished. The thought comes to mind that Sprague-Dawley rats have less resistance to the diabetic stress than do animals of the Long-Evans strain. This thought is in accord with the well-known hardiness of the Long-Evans strain.

SUMMARY

In diabetes caused by injecting alloxan into rats, the zona fasciculata of the adrenal glands was enlarged. Animals of the Long-Evans strain exhibited an increase in sudanophilia and in reactions indicative of ketosteroids. In Sprague-Dawley rats, the lipid and ketosteroid reactions were decreased. These changes are interpreted as indicating a condition of stress in which there is an increased demand for the carbohydrate-regulating hormone of the adrenal cortex.

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## A NOTE ON THE ESTROGENS IN THE BILE OF PREGNANT WOMEN[1]

CANTAROW et al. reported in 1943 that the estrogenic activity of liver bile ("C" fraction), obtained by duodenal intubation of women at full-term pregnancy, was about three times that of the blood. This finding was cited in support of a previously (1942) postulated theory of an enterohepatic circulation of estrogens. Following this clue, Pearlman et al. (1947) succeeded in isolating estrone in crystalline form from the bile of pregnant cows. The authors next turned their attention to the bile of pregnant women with the object of isolating and identifying the estrogens contained therein. Accordingly, over 20 liters of bile were assiduously collected by duodenal intubation of women who were in the last trimester of pregnancy.

Bioassay values for individual specimens of bile were found to be in line with those previously reported by Cantarow et al. (1943). Thus, for example,

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[1] This work was supported by grants-in-aid from the United States Public Health Service, under the National Cancer Institute Act, and from Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

three specimens of "B" bile (equivalent to gall-bladder bile) assayed for 4, 5 and 26.5 mouse units per ml. respectively; the corresponding "C" fractions (equivalent to hepatic bile) assayed respectively for 2, 5 and 12.5 mouse units per ml. These specimens when pooled with the "A" fractions (1.2 liters in all) assayed for 4 mouse units per ml. of bile.[2] Another specimen (0.45 liters) when similarly pooled assayed for 3 mouse units per ml. of bile. These figures are appreciably lower than those (average 22 mouse units per ml.) reported for gall-bladder bile of pregnant cows (Pearlman et al., 1947), which is not surprising because bile obtained by duodenal intubation is considerably more dilute than gall-bladder bile.

Procedures for the extraction and partitioning of the biliary estrogens were those previously (Pearlman et al., 1947) employed in this laboratory. The first step (extraction of the bile with butanol) gave a poor recovery of estrogenic activity in this instance, however. Thus, for example, a butanol extract of 1.2 liters of bile assayed for only 500 mouse units whereas about 4,500 mouse units were expected on the basis of bioassay performed directly

TABLE 1. DISTRIBUTION OF ESTROGEN* IN THE BILE OF PREGNANT WOMEN

| Volume of bile extracted (liters) | Batch number | Butanol extract (m.u.) | Free phenols (m.u.) | Conjugated phenols (after hydrolysis) (m.u.) |
|---|---|---|---|---|
| 13.9 | I | 9,500 | 2,000 | 14,600 |
| 8.9 | II | | 800 | 3,300 |
| 22.8 | I+II | | 2,800 | |

* Aqueous solutions of the butanol extracts and oily solutions of all other fractions were injected subcutaneously into adult spayed mice; a vaginal smear technique (Allen and Doisy, 1923) was used for bioassay.

on the bile; aqueous solutions of the bile or butanol extracts were injected subcutaneously into adult spayed mice for estrogen assay (Allen and Doisy, 1923). Such poor recovery of estrogenic activity was not encountered in the past when working with dog (Pearlman et al., 1948) or cow (Pearlman et al., 1947) bile under similar conditions. It is possible that labile estrogens are present in human bile; Werthessen, Baker and Borci (1948) claim to have detected labile estrogens in human and rat blood.

Tables 1 and 2 summarize the data on the distribution of estrogen in the bile of pregnant women. Both free and conjugated forms of estrogen are present whereas in the bile of pregnant cows the estrogen is almost entirely in the free form. The presence of estriol in human bile is suggested by the finding that the strongly acidic phenols of hydrolyzed bile extracts show some degree of estrogenic activity. However, due to the comparatively low content of estrogenic material in human bile, positive identification of this material could not be achieved. No estrogenic activity could be detected in the acid fractions of unhydrolyzed or hydrolyzed human bile. This makes it appear unlikely that acids of the doisynolic type are present in any significant

[2] It is worth mentioning for the sake of comparison that no estrogenic activity (i.e., less than 1 mouse unit per ml.) could be detected in the bile of non-pregnant women or of men.

TABLE 2. FURTHER FRACTIONATION OF PHENOLS

| Batch No. of bile | Nature of material | Weakly acidic phenols | | Strongly acidic phenols (m.u.) |
|---|---|---|---|---|
| | | ketonic (m.u.) | non-ketonic (m.u.) | |
| I | conjugated phenols (after hydrolysis) | 9,600* | | 5,000 |
| II | conjugated phenols (after hydrolysis) | 2,200 | <500 | 100 |
| I+II | free phenols | 800 | 330 | <500 |

* Total weakly acidic phenols.

amount; Miescher (1944) had made the interesting suggestion that such acids might be normally present in the organism.

An attempt to isolate neutral steroid hormones from butanol extracts of human pregnancy bile (about 23 liters) was abortive due to the lack of adequate amounts of material. Products of this type such as pregnanediol-3($\alpha$), 20($\beta$), pregnanol-3($\alpha$)-one-20 and etiocholanediol-3($\alpha$),17($\beta$) were recently isolated in this laboratory from the bile of pregnant cows (Pearlman and Cerceo, 1948).

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## NEW BOOK

Biochemistry and Morphogenesis. By Joseph Needham. Published Cambridge: at The University Press; New York: The Macmillan Company; 1942.—XVI+787 pp. $12.50.

Various circumstances have delayed our reviewing of Needham's great book. This delay of more than 5 years may be deplored as a precedent apt to inaugurate an era of sluggish digestion of scientific literature. On the other hand, a book like Needham's "Biochemistry and Morphogenesis," with an incredible amount of data, involved problems, and controversial discussions crowded into less than 700 pages and with more than 5,000 references, is hardly meant to be read from cover to cover within a few months. It may not, in fact, be a bad idea to use books of this type for a considerable period before attempting to review them.[1]

Similar to his 1931 "Chemical Embryology" Needham's new book aims at a correlation of chemical and morphological processes which occur during the development of the embryo. In "Biochemistry and Morphogenesis" the analysis reaches deeper strata, and the emphasis is on work published after 1931.

The arrangement of material is, in many respects, excellent. Some headings of chapters and subchapters are masterpieces of terse and yet tangible wording. On the other hand, Needham's attempt to divide the whole material into 3 major parts, entitled the Morphogenetic Substratum, The Morphogenetic Stimuli, and The Morphogenetic Mechanisms, is apt to produce a false impression of exactness. An arbitrary distribution of material among the 3 parts proved unavoidable. Respiration, including the energetics of differentiation (p. 574 ff.), is discussed in "Morphogenetic Mechanisms" while both nutrition and material combusted as source of energy (carbohydrate, protein, fat) are found under "Substratum" (p. 49). It seems to us that the metabolic properties of shell-enclosed eggs (pp. 48–63) and the standard mortality curves of chick embryos (pp. 63–66) belong to the "Mechanisms" rather than to the "Substratum," while the differential response to thyroxin of the fore-limb bud and the adjacent gill-tissue in tadpoles (p.448) would have found a more adequate place with the "Substratum" than with the "Stimuli." In other cases, the emphasis is, probably, a matter of personal preference.

Part 1 ("Substratum") contains a survey of recent data on the chemical constitution of eggs and of their nutritional material, with frequent references to older data in "Chemical Embryology." Needham suggests that Parts 1 and 3 of "Biochemistry and Morphogenesis" should be read in conjunction with the earlier book.

"The egg and its environment" is a representative chapter of Part 1. "Cleidoic" (i.e. closed) eggs, like those of birds, are completely endowed with

[1] I am grateful to my colleague Dr. S. B. Davis for comments and criticisms during the progress of this review, and I wish to acknowledge helpful personal communications by Prof. G. H. Bodine, Dr. E. W. Caspari, Prof. J. Holtfreter, and Prof. W. Landauer.

organic material, inorganic material and water; "non-cleidoic" eggs may either, from the beginning, contain all organic material, but receive inorganic material and water from the environment (e.g., sea-urchins and sand-crabs), or they may not be equipped with any nutritional material, but depend on the parental organism for continuous supply of everything (main representatives: mammals). These different conditions, and their relations to the aquatic, intermediate, or terrestrial environment are presented in a very clear way in Table 5. However, the table-heading "Evolution of the cleidoic egg" can hardly refer to *phylogenetic* evolution, since some species which are close together in ordinary taxonomy are found on opposite ends of the table, e.g., Scyllium (the dog fish) and Carcharias (a shark). Both are selachian fishes, but the first one is oviparous and the latter one viviparous, with striking differences in their biochemical development (Table 8).

Many readers will appreciate the data and pictures of the amazing tree frog whose tadpole stages are passed entirely within the egg. Treasures for endocrinologists are hidden at the bottom of page 43. Here Needham summarizes the work of the investigators at the Naples Biological Station who found the degree of dependence of selachian embryos on the mother to be correlated with a decrease in size of the maternal liver, the latter being controlled by hypophysis, thyroid and suprarenals.

Part 2 ("Stimuli") deals with inductors and organizers, with the relations of genes and organizers, and with gradients. The chapter which is entitled "A digression on plant galls" should convince any skeptic that chemical agents can indeed be responsible for the production of specific biological structures. However, up to the present, no one has been able to reproduce specific galls by any chemically defined substance, or even by a cell-free extract of an insect or its egg. Needham attributes this state of affairs to the relatively poor chemical technique which has been applied to the problem.

The subsequent 300 pages of Part 2 are centered around the "hierarchy of organizers in animal development." The 1st grade organizer, or "primary evocator," causes the appearance of the first axis of the embryo (gastrulation, formation of neural plate); examples of 2nd grade organizer effects are the induction of a lens by the eyecup, or of an ear-capsule by an ear vesicle; a 3rd degree organizer is at work in the induction of a tympanic membrane by the cartilage of the ear-capsule. The way in which the potentialities decrease with each subsequent grade of the "hierarchy" is illustrated by a model (Fig. 42, on p. 111) consisting of a series of cones on three different levels. A ball rolls from the top of the highest cone (=maximal potential energy) to its base, thus landing on the top of one of the next lower cones, from there rolls down to the base of this cone, etc. It is interesting to compare this austere model of equilibrium states with the picturesque railroad yard which was given in Needham's 1936 book "Order and Life."

About 100 of the 300 pages mentioned are devoted to amphibian morphogenesis, the classical subject of studies on induction and organizers. Unfortunately, the important fate-map of Vogt (Fig. 57) is reproduced on too small a scale, and the pictures illustrating movements (cell-streams) of vitally stained areas during amphibian gastrulation (Fig. 56) are still worse. It would have been worthwhile to render these basic pictures in colors. The black-white fate-maps of fishes (Figs. 179–181), although of secondary importance in this book, are printed in a more satisfactory way.

The germ-layers are disposed of with a few forceful arguments and the following conclusion: "Evidently the distinction between the germ-layers is a convenient morphological one with little or no physiological meaning" (p. 150).

In the chapters on biochemistry and chemistry of the primary organizer the inductive properties are discussed of living tissues, dead tissues, cell-free extracts, a number of impure substances and, finally, of well-defined substances like steroids.

Needham explains the disappointing "unspecificity" of the primary evocator (organizer) by the hypothesis that districts other than the dorsal lip of the blastopore may contain the "evocator substance" in an inactivated condition, and that the substance could be activated by unspecific mechanisms. A tentative list of such inactive complexes is given (pp. 206 and 207), and a way out of the dilemma is expected by closer attention to the question of the minimal effective dose. As a step in this direction a comparison is made between the effective doses of various evocating substances, of a cardiac drug, of several hormones and vitamins, and of two carcinogenics (Table 16, p. 184). Needham rightly emphasizes the difference between highly potent evocators such as 1, 2, 5, 6-dibenzanthrazene endo-$\alpha$-$\beta$ succinate, effective between 0.25 and 0.0025 mg./kg. wet weight, and weak evocators like nucleoproteins with an effective dose of 62500 mg./kg. wet weight. However, with respect to the other quite heterogeneous items of Table 16, a comparison is not only "a little difficult," as Needham puts it, but seems hardly possible to the reviewer.

A preliminary discussion of the metabolism of the gastrula anticipates the more detailed and final discussion in Part 3. A comparison between the evocator which induces the medullary plate (without cranial and caudal pole) and the auxin effect in plants is attempted in spite of the fact that the longitudinal growth of the cellulose walls in plant cells caused by auxin has no counterpart in the morphogenesis of animals.

In Needham's book abnormal organizer activities are considered responsible for three groups of formations which already in traditional human pathology have been treated as related phenomena, namely, twinning, teratomata, and malignant tumors. The greater part of these discussions (30 pages) are devoted to "organizers in cancer." Needham translates the old concepts of pathology into the new language. In his terminology the most important aspect of cancerous growth is that it is "an escape from the individuation field," whereby, in this case, individuation refers to any differentiation beyond the mere formation of a head- and tailless medullary plate. A large number of carcinogenic hydrocarbons and other substances are discussed with respect to their chemical and biological properties.[2] Diagram Fig. 138 shows 49 substances with their "overlapping domains" of different activities, for instance, 3, 4,-benzpyrene which is a primary evocator (neurogen), a carcinogen, and an estrogen. Unfortunately, colchicine is missing in the diagram, although present as No. 43 in the list of substances. Otherwise the diagram is excellent, and can be recommended for use in lectures.

[2] The much-discussed theory of the origin of malignant tumors from embryonic rests was abandoned not through "progress on biochemical lines" as Needham assumes (p. 240, footnote), but through recognition of the wide distribution of meristematic cells, i.e., tissue cells which, in the adult organism, maintain the ability of dividing.

Except for the determination of sex, little was known concerning the relations between genes and embryonic differentiation, when "Chemical Embryology" appeared in 1931. During the subsequent 10 years those relations have been investigated with so much success that they fill 90 pages of "Biochemistry and Morphogenesis." Genes which interfere with the action of the primary organizer are responsible for certain hereditary malformations of the heads in guinea pigs and chicks, and also for the short-tail malformation in mice, and the hereditary rumplessness in fowl. Genes interfering with the action of second-grade organizers are illustrated by examples like the genetic disturbances in the development of the eye or the limb-buds. A chapter "The origin of species specificity" contains examples of heteroplastic transplantations which document the fact of species specificity, but do not throw any light on its origin, as far as the reviewer can see.

The discussion on the role of the nucleus in embryonic development is based on hybridization, merogony, and parthenogenesis, with an excellent diagram (Fig. 205) and a very useful definition of terms (Table 24). A short survey of genes which control chemical processes is followed by a chapter on "nuclear inductors and nuclear metabolism." Here a series of splendid experiments with exchange of organs or fluids between different mutants of Drosophila is presented. Needham also gives a detailed description of studies on regeneration of Acetabularia, an alga which, in spite of its high degree of morphological differentiation and its size of 3 cms., contains only one nucleus. At the end of the discussion on genes and organizers the role of the sulfhydryl group and of glutathione is analyzed, mainly in the light of data obtained from large and small races of the same species, e.g., of rabbits and fowl. Needham points out that there are possible relations between glutathione and pituitary growth-hormone, but that the different rate of embryonic growth before the appearance of the pituitary remains unexplained, as the initial sizes of eggs are similar in small and large races.

The interrelations of genetical, endocrine and other factors are demonstrated by the frizzle condition in fowl, a hereditary malformation of feathers which disturbs the control of body temperature, and thus leads to changes of thyroid gland, food intake, intestinal length, blood picture, etc. Abnormal cartilage formation in rats, with its consequences, is another example showing the remarkable variety of phenomena which may be traced to one gene (Fig. 231).

The place of hormones in Needham's system will be of special interest to the readers of "Endocrinology." He classifies the products of endocrine glands as "the last of the three great inductor types," at least with reference to amphibial development. The two preceding types are the "true embryonic inductors" and the nuclear inductors derived from genes.

The imitation of gene-action by environmental factors ("phenocopies") is illustrated by several examples. The influence of genes and environmental temperature on the wing-pattern of the flour-moth (work of the Göttingen group) is discussed in detail, but the important relation between mitotic pattern and pigment production is not mentioned.

The last 50 pages of part 2 are devoted to organizer phenomena in insects, the metabolism in insect metamorphosis, and finally the determination in echinoderm development and metamorphosis. Needham reproduces some of the most important pictures from the studies of the Stockholm investigators

who demonstrated the double gradient system in the echinoderm egg. Improved recent experiments of Child and his collaborators are accepted as possible supports of the theory of *metabolic* gradients (pp. 496–497). Yet, Needham sees no reason why he should modify his negative attitude, as expressed in "Chemical Embryology," toward the experimental evidences of metabolic gradients, though he now praises Child for having introduced the concept (p. 605). An attempt to reconcile the organizer and the gradient theories concludes Part 2.

Part 3 ("Mechanisms") is introduced by an excellent chapter on the dissociability of the fundamental processes of ontogenesis showing how nuclear division can take place without cell division, cell division without nuclear division, differentiation of eggs without cleavage, etc. There are a few criticisms. On Table 28 "growth" is, without discussion, defined as "increase in spatial dimensions and in weight," although increase in volume, in dry weight, and in wet weight are dissociable processes, e.g., in the growth of tadpoles. In the list of fundamental processes the important migrations of cells and cell groups are missing. The dissociation of histogenesis from organogenesis is not made very clear (p. 514); malignant tumors could have been used as a striking illustration of this dissociation.

The unequal increase of different chemical components ("chemical heterauxesis") during embryonic development is illustrated in 12 charts and 4 tables, but there is little connection between these data and morphogenesis. The S-shaped growth curves of the whole body had been given much space in "Chemical Embryology," because of their resemblance to the curve of autocatalytic monomolecular reactions. In "Biochemistry and Morphogenesis" Needham devotes a few lines only to these curves stating rightly that their analysis "has been singularly lacking in fruitfulness or illumination." Nothing else could be expected, since mathematical resemblance alone does not indicate similar mechanisms; this was pointed out by G. Teissier (1928) and the reviewer (1933).[3]

The chapter on respiration deals with the early stages of eggs in echinoderms, and with various stages of embryonic development in arthropods, fishes, sauropsides and mammals. Particularly useful is a survey of recent pioneer work on the respiration of the grasshopper before, during and after the "diapause," which is a phase of morphogenetic dormancy. The discussion of carbohydrate metabolism covers, besides the data of other investigators, important contributions by Needham and his collaborators. The chapter on protein metabolism contains some pages on "The growth-promoting factor" of embryo juice as studied in tissue cultures. In the discussion on nuclein metabolism "complete synthesis of both phyto- and thymonucleic acid from non-cyclic precursors" is claimed for the embryos of birds and reptiles (p. 635). The meaning of "complete synthesis" is not clear to the reviewer; in any case the presence of nucleic acids in the unincubated egg is proved by the presence of its nucleus, though their amount may be too small for chemical detection. For lipin and sterol metabolism the reader is mostly referred to "Chemical Embryology," except for new work with tracer substances. Finally, the metabolism of various pigments is discussed.

The last 20 pages of Part 3 deal with "Protoplasmic Organization." Results of crystal-analysis of fibers are tentatively applied to the problems

[3] Arch. f. Entwicklungsmechanik, *130*, pp. 486–490.

of egg-cell structures. The characteristics of liquid crystals, or the para-crystalline state, are described in detail, and the biological significance of this state is emphasized. Among the proteins with highly anisometric molecules myosin (actomyosin, Szent-György) has moved into the center of interest. However, when "Biochemistry and Morphogenesis" appeared, only the first few studies on the relations between myosin and adenosinetriphosphatase had been published, including the 1941 paper by Needham, Shen et al. Under these circumstances some statements in "Biochemistry and Morphogenesis" seem almost prophetic, like that one on p. 670: "The probability is that molecular contractility plays a far greater part in development than embryologists have generally been willing to admit."

We have attempted to convey to the readers of "Endocrinology" an idea of the contents and the organization of Needham's book. The book is an expression of his creed that "structural alterations of the proteins are the basis of all morphology" (p. 605, footnote). Some bridges across "the secular gulf between morphology and chemistry" (p. XV) are, visibly, under construction on those pages which deal with "Protoplasmic Organization." In most of the other discussions the "bridges" still are in various stages of planning in spite of all the interesting correlations between biochemical and morphological phenomena of ontogeny. Needham's book gives an excellent account of the state of affairs.

Everyone will appreciate the fact that Needham tried to limit his gigantic task by excluding functional physiology as much as possible (see p. 643 footnote, on embryonic movements and reflexes). Yet, embryonic cells have to satisfy requirements for maintenance besides those for development; as G. L. Streeter used to put it, "embryonic tissues are open for business during alterations." This consideration would have been helpful in Needham's discussions of respiration and metabolism.

It is obvious that not all of the numerous data and techniques reported in "Biochemistry and Morphogenesis" could be handled with equal care. In connection with the regional respiration and glycolysis of gastrulae various micro-manometric techniques are compared in adequate detail; on the other hand, the absence of "the characteristic protein curve" in intact living sea-urchin eggs is stated (p. 656) without mentioning the limitations of spectrography in heterogeneous systems. Publications on tissue cultures are quoted without technical comments; a brief summary of these techniques is promised on p. 628, but not given. Unnecessary arguments are the result, as in the case of the lactogenic hormone. In the pigeon's crop in situ, the mitotic activity of the epithelium is increased by lactogenic hormone, but, according to Needham (p. 76), "this action could not be reproduced in vitro." The original papers show that the work in situ is valid, while the work in vitro is not: embryo juice had been added to all cultures in vitro so that the highest growth-rate possible was already obtained in the controls.

Sometimes, Needham's lack of interest in non-chemical procedures has led him to select poor papers when better ones were available. Abnormal turning of the 2–3 day-old chick embryo on its right side instead of its left occurs in 2 to 6% according to all authors who observed the embryo through an opening in the shell. The only paper quoted by Needham (p. 65) gives 0.163%, a pseudo-accurate figure based on mere candling of eggs on the 5th day (which is too late anyway).

The bibliography is an especially useful part of the book. Most of the references proved to be correct when checked. Only a few publications are misquoted. The paper by Crosman, quoted on p. 66, does not deal with the absorption of dead embryos in rodents, neither does the paper by Ancel et Vintemberger, quoted on p. 218, say anything about the possible effects of incident light on the plane of symmetry. On p. 629 Needham reports that the inhibition by "lipins" of the growth-promoting effect of embryo extract is reversed by heparin, while that is not so according to the original paper by E. Mayer.

The numerous graphs are very helpful. If their authors were stated in the legend, it would be clearer which graphs were made by Needham, modified by him, or reprinted unchanged. The "Glossary" gives a number of important definitions. However, "Gestalt" is antonymous to "the mere sum of parts," not to "surroundings." Could Driesch's old terms "prospective significance" and "prospective potency" not be replaced by routine potency, spare potency and total potency? The "Mitogenetic rays" are, to our deep satisfaction, on Needham's list of "Terms and concepts the use of which is not recommended."

The alphabetic index prepared by Margaret Miall is satisfactory with some exceptions. It is difficult to find the various places where axes and poles are mentioned. For large items like "Chick embryo and tissues" (30 different pages indexed, 2 of them wrong), and "Sea-urchin" (20 different pages indexed) some subindexing would have been useful.

Many interesting historical and linguistic remarks are scattered in the book. The reviewer takes it for granted that the Chinese sentences are correct, but would have preferred the absence of wrong German like the "erster Nährung" (instead of "erste Nahrung") on p.15. The verses which Needham ascribes to Goethe (p. 231) are from the "Lied von der Glocke" by Schiller. Names of American authors like Chaikoff and Romanoff should not have been altered into Chaikov and Romanov, irrespective of Needham's linguistic arguments (p. 692).

As stated before, it is evidently impossible to write great synthetic books like "Biochemistry and Morphogenesis" without a certain number of errors. Original papers are, as usual, to be consulted whenever a special point is of interest to the reader. In spite of all criticisms "Biochemistry and Morphogenesis" is an admirable and very useful book. We hope that it will help in attracting brilliant young workers to the promising field of experimental embryology.

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# ASSOCIATION NOTICE

## ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

## POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

# ASSOCIATION AWARDS FOR 1949

## THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

## THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

## THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

# ENDOCRINOLOGY

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# STUDY ON THE MECHANISM OF PICROTOXIN-INDUCED OVULATION IN THE RABBIT[1]

S. CHAS. KASDON[2]

## PICROTOXIN INDUCED OVULATION

THE INDUCTION of ovulation by Marshall and Verney (1936) through electrical stimulation of the hypothalamic region was a major step forward in the study of the mechanism of ovulation. They reported (1938, 1939) that picrotoxin in convulsant dose (1 mg./kg. of body weight) caused ovulation in 6 of 21 rabbits tested. With few exceptions all the animals showed signs of follicle growth or hemorrhagic follicles but ovulation occurred in only 22 per cent of the animals tested. Usually the normal number of 3 to 5 ovulating follicles was found but the latent period between picrotoxin stimulation and ovulation was from 24 to 48 hours as compared to the normal period of 10 hours between copulation and ovulation. A smaller dose of picrotoxin (0.8 mg./kg.) in the presence of atropine caused violent convulsions, but none of the rabbits so treated ovulated. Since this dose of picrotoxin did not produce ovulation in the absence of atropine, it was felt that the failure was due to the smaller dose of picrotoxin. They interpreted this as an indication that no correlation existed between the convulsant activity of picrotoxin and its effect on the ovaries. Further, Makepeace (1938) found that atropine did not prevent ovulation postcoitum, but he did not use isolated rabbits in this study. Other convulsants were employed—strychnine, coriamyrtin—and general excitants—apomorphine, beta tetrahydronaphthylamine and ergometrine—but proved equally inactive. Marshall, Verney and Vogt (1939) inferred that the "absence of response might be due to failure in finding the optimal conditions for their action."

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[1] This work was completed under the direction of Dr. F. F. Snyder at the Chicago Lying-in Hospital, University of Chicago Clinics, University of Chicago, Chicago, Illinois.

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Copper salts administered intravenously were found more predictable in the production of ovulation in rabbits by Fevold, Hisaw and Greep (1936). They felt that these salts as well as picrotoxin probably caused ovulation by augmenting the effect of the gonad stimulating hormones in the blood to the level essential for ovulation. On the other hand, Bischoff (1938) felt that the injection of so toxic a substance as copper caused ovulation through nervous stimulation. The problem was resolved by Brooks, Beadenkopf and Bojar (1940) who sectioned the pituitary stalk in rabbits and found that 10–15 mg. of copper acetate—a dose universally effective in producing ovulation in the intact rabbits which survived the injection—failed to produce ovulation. When the hypophyseal stalk was cut in 11 animals, injections of picrotoxin also failed to result in ovulation. Metrazol was similar in its ovulatory action but less effective than picrotoxin. Convulsant doses of insulin, benzedrine and ephedrine chloride all failed to induce ovulation. They too felt that conditions must be favorable for ovulation to occur before picrotoxin and metrazol could be effective, but were unable to explain why such dissimilar substances should cause ovulation to occur in the rabbit when various other excitants and convulsants were inactive in this respect.

An excellent review of the pharmacological activity of picrotoxin was published by Dille (1938, 1939) and will not be discussed here. The specific antagonistic action of barbiturates for picrotoxin was described by Swanson (1932) and has been reviewed by Tatum (1939). That barbiturates themselves do not interfere with ovulation has been adequately demonstrated by Brooks and his colleagues (1940). This protective action of barbiturates makes it possible to use picrotoxin in several times the effective ovulating dose without sacrifice of the animals. It could then be established whether the effect of picrotoxin on ovulation is directly related to its convulsant properties or to the quantity of picrotoxin injected into the rabbit.

The convulsant action of picrotoxin was early studied by Pagniez (1929). It was found to have a depressant effect upon the rhythmic activity of leech muscle (Gessner, 1932), as well as a vasoconstrictor action not antagonized by ergotamine (Gessner, 1928). Miller and Spiegel (1939) decorticated rats and administered picrotoxin to establish the part played by the cerebral cortex but found it just as effective a convulsant in the decorticate animals. Schriever and Perschmann (1936) localized the seat of action to the midbrain which is significant in that the hypophysis is found to be an essential link in the catenary system whereby picrotoxin causes ovulation.

Further, the fact that rabbits which have just littered are highly susceptible to mating makes it possible to obtain animals "primed" for ovulation. The ovary of the postpartum rabbit was found "primed" for ovulation by Friedman (1932) and responded to the injection of one minimal effective dose of extract of pregnancy urine. It was felt

that the use of puerperal animals would make it possible to satisfy to some extent the "favorable conditions" for ovulation that both Marshall and Brooks and their colleagues have stressed as essential in the ovulatory activity of picrotoxin.

## METHOD

Mature but virgin female rabbits, healthy and well fed, were isolated for at least 20 days before use in order to control the occurrence of single hemorrhagic follicles frequently found in such animals. Phenobarbital was used in 10 per cent aqueous solution and the picrotoxin was used in 20 per cent alcoholic solution. Laparotomy was performed under drop ether anaesthesia with phenobarbital used as a basal anaesthetic in some animals and oophorectomy was performed in all suspicious cases and in most of the others. All laparotomies were performed 48–72 hours after the injection of the picrotoxin. The animals injected after parturition received picrotoxin intravenously within 12 hours of the time the litters were dropped. Tissues were fixed in 10 per cent formalin and stained with hematoxylin-eosin. In every case the diagnosis of ovulation was established by microscopical study of the tissues. Hemorrhagic follicles were not recorded as proof of ovulation. The importance of identifying ovulation by means of microscopical examination of the ovary or by actual recovery of the ova from the tubes has been stressed by Lewis and Wright (1935).

## RESULTS

As a control experiment, the work of Marshall, Verney, and Vogt (1939) was repeated using the dosage and technique which they found

TABLE 1. PICROTOXIN INJECTIONS IN ISOLATED VIRGIN DOES

| Picrotoxin mg./kg. i.v. | Hyperactivity + and Convulsions ++ | Ovulation |
|---|---|---|
| 1.0 | ++ | − |
| 1.0 | + | − |
| 1.0 | ++ | + |
| 1.0 | ++ | + |
| 1.0 | + | − |
| 1.0 | + | − |

successful in causing ovulation in the rabbit. Accordingly, a series of 6 rabbits received 1.0 mg./kg. of picrotoxin intravenously (sc. Table 1) but in spite of the production of convulsions in half of them, ovulation was found at laparotomy in but 2. This failure to induce ovulation in more of the normal isolated does at levels of picrotoxin found adequate in some rabbits under identical conditions serves but to emphasize the uncertain chain of events that is apparently essential for the occurrence of picrotoxin-induced ovulatión in the rabbit.

In the next series the rabbits used had dropped their litters within 12 hours of the time picrotoxin was administered in order to minimize as much as is possible by this means variation in susceptibility for

ovulation in the host. Twenty-four rabbits were employed with results shown in Table 2. The first 10 animals received 1.0 mg./kg. of picrotoxin intravenously and none ovulated in spite of severe convulsions which occurred in half of them. The remaining 5 rabbits in this group showed great excitability. The dose was then raised to 1.5 mg./kg. of body weight injected intravenously, a dose well above the level found capable of producing ovulation by Marshall, Verney and Vogt. All of these rabbits developed severe convulsions and 2 of the 14 so treated expired in their course. In 2 rabbits the presence of ovulation was demonstrated microscopically. Thus, ovulation occurred in but 12.5 per cent of the rabbits surviving the convulsions. It is significant that in spite of a larger dose of drug, in spite of the

TABLE 2. PICROTOXIN INJECTIONS IN "PRIMED" RABBITS 12 HOURS POST-PARTURITION

| Picrotoxin mg./kg. i.v. | Hyperactivity + and Convulsions ++ | Ovulation |
|---|---|---|
| 1.0 | ++ | expired |
| 1.0 | + | – |
| 1.0 | ++ | – |
| 1.0 | + | – |
| 1.0 | ++ | – |
| 1.0 | + | – |
| 1.0 | ++ | – |
| 1.0 | ++ | – |
| 1.0 | + | – |
| 1.0 | + | – |
| 1.5 | ++ | – |
| 1.5 | ++ | + |
| 1.5 | ++ | – |
| 1.5 | ++ | – |
| 1.5 | ++ | – |
| 1.5 | ++ | expired |
| 1.5 | ++ | – |
| 1.5 | ++ | – |
| 1.5 | ++ | + |
| 1.5 | ++ | expired |
| 1.5 | ++ | – |
| 1.5 | ++ | – |
| 1.5 | ++ | – |
| 1.5 | ++ | – |

presence of convulsions, and in spite of rabbits "primed" for ovulation in the postparturitional state, such a small number were found to ovulate.

In the third group of barbiturate protected animals, the picrotoxin was administered intravenously in doses much larger than those administered by Marshall, Verney, and Vogt. Even with the relatively small dose administered intravenously 20 per cent of their unprotected rabbits expired during the convulsions. The minimal lethal dose of picrotoxin for rabbits has been established by Swanson and Chen (1936) as 2.5 mg./kg. when administered intravenously and only slightly higher when given subcutaneously. The minimal lethal dose for phenobarbital in rabbits is in the neighborhood of 150

mg./kg. From Table 3 it can be seen that from 2 to 6 times the dose of picrotoxin used by Marshall or by Brooks and their colleagues was administered to the rabbits protected against such a lethal dose by phenobarbital without evidence of ovulation at laparotomy. Convulsions occurred only in the 3 animals receiving the largest dose of picrotoxin, 2 of which expired during convulsions. In the one rabbit surviving the convulsions no evidence of ovulation was found at laparotomy. The significance of this series is that picrotoxin in a dose 2 to 6 times that found capable of inducing ovulation was completely ineffective here.

TABLE 3. PICROTOXIN INJECTIONS IN VIRGIN ISOLATED RABBITS PROTECTED BY PHENOBARBITAL

| Picrotoxin mg./kg. i.v. | Phenobarbital mg./kg. Subcutaneously | Hyperactivity + and Convulsions ++ | Ovulation |
|---|---|---|---|
| 2.0 | 100.0 | + | – |
| 2.0 | 100.0 | + | – |
| 3.0 | 120.0 | + | – |
| 6.0 | 120.0 | + | – |
| 6.0 | 160.0 | ++ | – |
| 9.0 | 120.0 | ++ | expired |
| 12.5 | 66.5 | ++ | expired |

## DISCUSSION

The evolution of the process whereby ovulation occurs is still in the period of development but the last 20 years have seen important advances made. With the use of both normal and hypophysectomized rabbits a definite relationship between ovulation and the anterior lobe of the pituitary was demonstrated (Bellerby, 1934). The early study of Fee and Parkes (1929) on the rabbit showed that hypophysectomy within one hour of copulation inhibits ovulation, but later than one hour does not. Friedgood and Pincus (1935) showed that the anterior hypophysis was apparently directly responsible for the initial maturation of ovarian follicles through its gonadotropic properties. That the sympathetic nervous system is involved is accepted, but the fact that ovulation could occur in rabbits with the thoraco-lumbo-sacral sympathetic system (Hinsey and Markee, 1932) as well as the cervical sympathetics interrupted (Haterius, 1934) places this system early in the series of events which lead eventually to ovulation. It is quite evident that the anterior pituitary lobe is a direct effector by a humoral route through which ovulation occurs in the rabbit.

In this study the ability of picrotoxin to induce ovulation in the rabbit was studied, and several significant facts elucidated. In one series picrotoxin in a dose several times that found adequate to induce ovulation in the rabbit was administered to does protected by phenobarbital against the lethal quantities injected. Ovulation resulted in none of the rabbits including one which survived severe con-

vulsions following a dose 6 times that found effective and more than twice the minimal lethal dose. The fact that ovulation did not obtain although there can be no question of the fact that the picrotoxin was physiologically effective is significant. The barbiturate was exonerated in antagonistic effect by the report of Brooks, Beadenkopf and Bojar (1940). This suggests that the ovulatory activity of picrotoxin is not directly related to the quantity of drug in the body. It is also noteworthy that frequently in this series of rabbits a moderately severe hyperemia of the uterus and tubes was found. Although the number of animals used is admittedly small, it is demonstrated that larger doses of picrotoxin are certainly no more effective than those one-half to one-sixth as great found irregularly capable of producing ovulation.

A control group of animals received a dose of picrotoxin reported to be effective in causing ovulation to occur in less than one third of the rabbits tested and one third of these were found to ovulate. That it can do so at times is undeniable, but it is apparently difficult to predict the ovulatory effect of picrotoxin. That convulsions in themselves are not sufficient to induce ovulation in the rabbit is apparent from the numerous failures following convulsions caused by insulin, strychnine, coramine and electrical stimulation. In addition, although ovulation following picrotoxin injection occurs in convulsant doses, many rabbits which experienced convulsions did not ovulate.

In a third series of rabbits injected after parturition which were in an optimal "primed" condition for ovulation but 2 of 21 rabbits (10 per cent) surviving a dose considered effective ovulated. This level of successful activity is lower than that reported by Marshall and by Brooks and their colleagues as well as in the control series of rabbits here reported. The failure to induce ovulation more frequently in these "primed" rabbits was somewhat unexpected, and serves to make even more obscure the "favorable conditions" under which picrotoxin can produce ovulation. "Priming" was also attempted by Brooks, Beadenkopf and Bojar by injection of sub-ovulating doses of pituitary follicle-stimulating hormone (FSH). This did not increase the frequency of ovulation following picrotoxin injections in the rabbit.

In spite of the small numbers of animals employed in these experiments it can be stated that picrotoxin induced ovulation in the rabbit occurs unpredictably at convulsant levels in approximately one-ninth of the animals tested. The fact that 2 to 6 times the effective dose did not induce ovulation suggests that it is not the quantity of the drug alone which determines its ovulatory activity. However, it is just possible that, in spite of evidence to the contrary, quantities of barbiturates sufficient to protect the rabbits against levels of picrotoxin greater than the lethal dose may interfere with its ovulatory activity. Finally, it is apparent that lack of susceptibility of the host

to ovulation, as determined by the post-parturitional "primed" state of the rabbit for ovulation, is in itself not a final determinant of picrotoxin-induced ovulation in the rabbit.

## SUMMARY

The induction of ovulation in the isolated rabbit doe was found to occur in a total of 4 out of 27 animals (15 per cent) surviving the administration of picrotoxin in reported adequate dose.

Two to 6 times the effective ovulatory dose of picrotoxin fails to induce ovulation in rabbits protected against the lethal quantity of picrotoxin by phenobarbital.

The frequency of successful induction of ovulation with picrotoxin is not increased by augmenting the susceptibility of the host by means of the ovulation-primed state following parturition.

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# A NEURAL FACTOR IN THE MECHANISM BY WHICH ESTROGEN INDUCES THE RELEASE OF LUTEINIZING HORMONE IN THE RAT[1]

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## INTRODUCTION

In polyestrous animals such as the rat, mouse, and guinea pig, which ovulate spontaneously, estrogen stimulates the release of luteinizing hormone from the hypophysis (Fevold, 1939; Hellbaum and Greep, 1946; Hisaw, 1947; Everett, 1947, and Bradbury, 1947). The liberated LH[2] proceeds to induce preovulatory swelling, ovulation, formation of new corpora lutea and, in pregnant or pseudopregnant rats, cholesterinization of existing active corpora lutea (Everett, 1947). The spontaneously ovulating species do not require an overt neural stimulus, such as is presumably initiated by copulation, for the release of the gonadotrophic hormone. Nor is it established that coitus hastens ovulation in these animals. In this respect they differ from such species as the rabbit, cat and ferret, in which the release of an ovulating surge of LH normally occurs only after copulation. Conversely, estrogen alone will not stimulate discharge of LH in the animals of the latter type (Bachman, 1936; Dury and Bradbury, 1943).

There is considerable evidence that, in the spontaneously ovulating group, estrogen acts directly upon the hypophyseal cells with no intermediation whatever by the nervous system. This evidence derives from three sources: (1) Hypophyseal grafts, which are of necessity removed from normal neural relationships, have been reported to maintain the integrity of sex function in hypophysectomized rats (Greep, 1936; May, 1937). (2) Section of the pituitary stalk, which severs the nerve fibers entering the hypophysis, has been asserted to be consistent with normal sex function in the guinea pig (Dempsey, 1939; Dey, 1943), in the rat (Dempsey and Uotila, 1940; Dempsey and Searles, 1943) and in the dog (Brekenridge and Keller, 1948).

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[1] This study was supported in part by a grant from the Duke University Research Council.

[2] LH is used in the conventional sense (cf. Hisaw, 1947) although its effects here may largely express quantitative rather than qualitative differences in gonadotrophic secretion.

(3) Cytological changes, induced by massive doses of estrogen, occur in hypophyseal grafts (Desclin and Gregoire, 1936; Martins, 1936, a; Hohlweg, 1936; Phelps, Ellison and Burch, 1939) and in stalk-sectioned pituitaries (Westman and Jacobsohn, 1938, a; Uotila, 1940) as readily as in non-operated hypophyses (Hohlweg, 1934; Severinghaus, 1939; Baker and Everett, 1947).

A nervous element in gonadotrophic control is suggested by evidence from the same sources: (1) More or less impairment of sex function has been reported in guinea pigs (Schweizer, Charipper and Haterius, 1937) and in rats (Westman and Jacobsohn, 1940) which have been hypophysectomized and implanted with pituitary grafts. (2) Mild to severe symptoms of gonadotrophic upset have been claimed as sequelae of section of the pituitary stalk (Richter, 1933; Westman and Jacobsohn, 1937, 1938; Herold, 1939; Herold and Effkemann, 1938; Westman, Jacobsohn and Hillarp, 1943; Brolin, 1945). (3) Certain cytological changes in the hypophysis appear to require a neural stimulus. Castration cells which ordinarily form in response to gonadectomy do not appear in grafted hypophyses (Hohlweg and Junkmann, 1932; Desclin and Gregoire, 1936; Martins, 1936, b; Westman and Jacobsohn, 1940) nor in pituitaries whose stalks have been severed (Westman and Jacobsohn, 1938, a).

The investigators whose results are so diametrically opposite have criticized each other's techniques. For instance Uotila (1940) suggests that Westman and Jacobsohn's stalk-section operation damaged the anterior hypophysis, whereas Westman, Jacobsohn and Hillarp (1943) point out that Uotila's stalk-sections were incomplete. Obviously a procedure which would cause no structural damage to either nerves or blood vessels and yet would prevent the effects of nervous activity from reaching the hypophysis would be a highly desirable method to resolve the controversy. Such a technique has recently proved effective in the rabbit in preventing the neurogenic stimuli attending copulation from releasing LH (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949). The technique consists of injecting adrenolytic or anti-cholinergic agents into the animal at the appropriate time. The rabbit evidence indicates that the neurogenic stimulus for the release of LH includes both cholinergic and adrenergic components, the former initiating the latter (Sawyer, Markee and Townsend, 1949). That the blocking drugs, Dibenamine and atropine, inhibit ovulation by preventing the stimulus for LH release from reaching the hypophysis, rather than by interfering with the liberation of the hormone from the gland itself, is indicated by this fact: delaying injection less than a minute post coitum permits ovulation to proceed in an unimpaired manner although at least an hour is consumed in the discharge of LH (Fee and Parkes, 1929).

The blocking technique has therefore been applied to the problem of whether estrogen stimulation of the hypophysis, leading to the release of LH in a spontaneously ovulating species, represents a direct

effect of estrogen on hypophyseal cells or an indirect stimulation via the nervous system. The study takes advantage of another new technique, one which readily reflects the release of LH, and to a certain extent registers its quantitative aspects and time relationships, in response to estrogen in the rat (Everett, 1947). If female rats are injected with estrogen on the fourth day of pregnancy, they regularly ovulate on the second night thereafter and a rich deposition of cholesterol occurs in the corpora lutea at about the same time. LH injected on the fifth day of pregnancy causes both ovulation and cholesterol storage within 18 hours even if the hypophysis is removed at the time of injection.

In the present study we have administered blocking agents in conjunction both with estrogen and with LH. The results to be presented below indicate strongly that the estrogen stimulus for the release of LH is exerted primarily upon the nervous system and only indirectly on the hypophysis itself. The neurogenic stimulus, initiated or facilitated by estrogen, contains both cholinergic and adrenergic components as in the rabbit. Neurogenic stimuli effective in releasing sufficient LH to induce ovulation are not initiated, under the conditions of the experiment, until at least 20 hours after the administration of estrogen. Part of the results of the present work have already been reported in abstract form (Sawyer, Everett, and Markee, 1948; Everett, Sawyer and Markee, 1948).

## MATERIALS AND METHODS

Sixty sexually mature female albino rats of the inbred Vanderbilt strain (a derivative of the Osborne-Mendel colony) were used in this study. Each was mated during proestrus, as determined by daily vaginal smears, with a known fertile male. If sperm were present in the smear the following morning, pregnancy was dated with that morning as zero time.

Estradiol benzoate was administered in sesame oil by subcutaneous injections. The standard dose, at a concentration of 0.33 mg./ml., was 50 $\mu$g. (0.15 ml.). Two samples of purified sheep hypophyseal LH[3] were used (*P 118* and *15HM3*). Doses of 3 mg. of either material in aqueous solution (10 mg./ml.) were known to be fully effective in ovulating pregnant rats and producing cholesterinization of their corpora lutea within 18 hours.

Solutions of Dibenamine hydrochloride and atropine sulfate (USP XIII, Mallinckrodt) were prepared from the salts just prior to injection. Dibenamine was first dissolved in 95% alcohol at a concentration of 100 mg./ml. and this solution was diluted to a final concentration of 10 mg./ml. with unbuffered Ringer-Locke solution. With the use of a tuberculin syringe and a

[3] These preparations of LH were generously contributed by Dr. R. K. Meyer, University of Wisconsin. They were assayed in his laboratory for both FSH and LH activity. In *P 118* no FSH was detectable in 3.3 mg. (0.5 gm. equivalent of the whole dried gland), yet the same quantity augmented ovarian stimulation by a standard FSH preparation (average ovarian weight in LH and FSH: 60 mg., contrasted with 45 mg. produced by FSH alone). In *15HM3* very slight FSH activity was found in 5 mg. (0.5 gm. equivalent), while the same quantity with FSH gave marked augmentation (average ovarian weight: 115 mg.).

27 gauge needle the Dibenamine solution was injected very slowly (about 0.5 ml./minute) into the small saphenous vein of the unanaesthetized rat. The injection was performed slowly because the amount of the drug tolerated on slow administration is much greater than on rapid injection (Nickerson and Goodman, 1947). The foot and leg were prepared by swabbing with 70% alcohol and shaving off the hair, and the vein was usually entered below the ankle. The injection technique requires two people, one to hold the rat, previously wrapped in a towel, and the other to perform the injection. The site is an excellent one for highly effective intravenous injections because the outline of the vein is sharply demarcated, and the needle may be held in the vein by the operator's hand which is clasping the rat's foot.

Preliminary experiments to determine the optimal dosage of Dibenamine employed 15 adult male rats. They were injected with doses ranging from 20 to 40 mg. per kg. For reasons to be presented below, 30 mg./kg. was adopted as the standard blocking dose although, as we shall see, it is not completely effective in blocking estrogen-induced liberation of LH.

Atropine sulfate was dissolved directly in Ringer-Locke solution. Preliminary attempts to find an intravenous blocking dose revealed that rats would tolerate a dose of 50 mg./kg. while 60 mg./kg. was lethal to one and almost fatal to 2 others out of 4. The intravenous injection of 50 mg./kg. atropine, however, failed to block estrogen-induced ovulation, probably because its effect was too short-lived. Therefore, the subcutaneous route was subsequently employed. The solution was made up as 70 mg./ml.; at this concentration it required only a simple calculation to administer the effective subcutaneous dose, 700 mg./kg. (Holck, 1942)—one ml. was injected per 100 mg. of body weight.

In a typical experiment Dibenamine was injected intravenously at 11 A.M. on the fourth day of pregnancy, and one hour later estradiol benzoate was injected subcutaneously. In another series of experiments, estrogen was administered at noon on the fourth day of pregnancy and Dibenamine at 8 A.M. on the fifth day. Atropine was injected subcutaneously, always at about 8 A.M. on the fifth day of pregnancy. LH was injected at 11 A.M. or noon on the fifth day. The animals were usually killed with illuminating gas and autopsied on the morning (occasionally early afternoon) of the sixth day. The ovaries were examined in physiological saline under a dissecting microscope for external evidences of stimulation by LH: hypertrophy, hyperemia or rupture of the largest set of follicles and a shift in the relative lipid content of interstitial tissue and corpora lutea of pregnancy. Tubal ova were visualized by removing the ampullar end of the oviduct and flattening it in saline with a cover slip on a microscope slide.

One or 2 corpora lutea were removed for fixation in 10% formol and the remainder of the ovary was fixed in Zenker's solution. The Zenker-fixed material was embedded in paraffin, sectioned at $10\mu$, or occasionally at $15\mu$, and stained with Harris' hematoxylin and a modification of Mallory's triacid stain (Everett, 1943). The formalin-fixed pieces were sectioned on the freezing microtome at $30\mu$ and were studied for cholesterol content by the Schultz technique (Everett 1945, 1947).

## RESULTS

*The optimal dose of Dibenamine.* In preliminary tests of survival at various Dibenamine dosage levels, employing 15 male rats, the

following results were obtained (the dosage is followed by the proportion which survived): 20 mg./kg., 1/1; 25 mg./kg., 5/5; 30 mg./kg., 4/6; 35 mg./kg., 1/2; and 40 mg./kg., 0/1. The animal which received the 40 mg./kg. dose and one of those which was injected with 30 mg./kg. died almost immediately; the others which failed to survive succumbed during the first night. The rest lost from 5 to 15% of their body weight during the next 2 or 3 days, the loss being attributable at least in part to a temporary loss of appetite.

Inasmuch as 25 mg./kg. was the highest dose which all the rats survived, this dosage was injected in the first attempts to block estrogen-induced ovulation. Of 5 female rats receiving this dose of Dibenamine prior to estrogen administration (5 μg. estradiol benzoate) on the fourth day of pregnancy, 3 were found at autopsy to have ovulated. The 5 μg. injection of estradiol benzoate was not completely effective in inducing ovulation: when this dose was administered in the absence of Dibenamine, only 2/4 rats ovulated. However, assuming 5 μg. estrogen to be a completely adequate stimulus, 25 mg./kg. of Dibenamine would be only 40% (2/5) effective in blocking estrogen-induced ovulation. Therefore a dose of 30 mg./kg. of Dibenamine was adopted for further experiments which assayed to block the estrogen induced liberation of LH.

*Effects of Dibenamine and atropine not directly concerned with gonadotrophic function.* No attempt was made to study in detail the pharmacological actions of Dibenamine and atropine. We were, however, impressed by the constancy of certain sequelae of drug administration to the female rats in attempted-blocking experiments. Some of these observed responses will be reported here.

Within a few seconds after an intravenous injection of Dibenamine (10 mg./ml.) was begun, a predictable jerk of skeletal musculature would all but dislodge the needle. From that instant until the termination of injection (the dose of 30 mg./kg. required 0.60 ml. for a 200 gm. rat, and the time consumed was more than a minute) the musculature remained rigid or trembled. Within the next 2 or 3 minutes, following release of the rat to its cage and continuing for 5 to 10 minutes, spontaneous contractions of the extensor trunk musculature would pull the rat to an upright posture on its hind legs, from which position it would fall backwards or sidewise. The sequence would be repeated several times, after which the rat would remain prone and quiescent for some hours. Even the following day the rat usually was lethargic and often felt cold to touch. It was observed not to feed during the first several hours after injection and undoubtedly lost weight, as did the males in preliminary experiments. The dose of 30 mg./kg. of Dibenamine was somewhat better tolerated by female rats in blocking experiments than by the males in preliminary injections, perhaps due to more careful, slower administration to the females. Only 3 females of 45, which received Dibenamine in the current series, expired before the intended hour at which each experiment was to be terminated.

Certain observed reactions to Dibenamine might be classified as cholinergic phenomena. Within a few minutes after injection there was usually profuse salivation, occasionally so copious that it interfered with breathing. Within an hour after injection there was often evidence of chromodacryorrhea, porphyrin secretion by the Harderian glands, appearing as "bloody" tears. The porphyrin nature of the secretion was ascertained in the present experiments by its red fluorescence in ultraviolet light. Chromodacryorrhea is a cholinergic response and has been used as a test for the presence of acetylcholine (Tashiro et al., 1940). The pupillary constriction, however, which was so constant in the Dibenamine-treated rabbit (Sawyer et al., 1947), was only occasionally observed in the rat. Another fairly common occurrence was the excretion, within the first hour after injection, of bloody urine. The pigment in the urine was found, on spectroscopic analysis, to be actually oxyhemoglobin. The presence of hemoglobin in the urine, in contrast with secreted porphyrin in the tears, is indicative of extensive destruction of erythrocytes by Dibenamine.

A quite constant effect of Dibenamine in conjunction with estrogen (but not with LH) was the subcutaneous deposition of a mucoid material in the thigh, inguinal region and sometimes the whole abdomen on the side of the Dibenamine injection site. The mucoid was readily located even without making an incision because it separated the skin and the underlying muscle in such a way that the skin appeared translucent. As will be mentioned below there appeared to be a positive correlation between the abundance of the mucoid and the degree of blockage of estrogen-induced release of LH.

Subcutaneous injection of atropine sulfate (700 mg./kg.) was followed within a minute or two by maximal dilatation of the pupil. Before autopsy the external abdominal wall was observed to be wet with urine and this incontinence was related at autopsy to a maximally dilated, atonic bladder. The skin and connective tissue at the site of injection were very hyperemic and the underlying muscular wall was rigidly contracted.

*Injection of estrogen alone on the fourth day of pregnancy.* Subcutaneous injection of 50 $\mu$g. estradiol benzoate into 8 female rats on the fourth day of pregnancy resulted in ovulation and cholesterol storage in all 8 as observed at autopsy on the sixth day (figure 1, A). All of the pregnancies were interrupted: none of the uteri were gravid on the sixth day. Tubal ova were visualized and rupture points of new corpora lutea were observed in each case. The corpora lutea of pregnancy all appeared fatty and all gave strongly positive (++) Schultz tests. These observations confirm the findings of Everett (1947). In figure 1, A (as also in 1, C, and 2, D) the + Schultz reaction shown in the fifth-day corpus luteum is based directly on the earlier work; none of the 8 estrogen-treated females in the present study was autopsied before the sixth day.

*Injection of both Dibenamine and estrogen on the fourth day of pregnancy.* The results of injecting Dibenamine into 12 female rats one

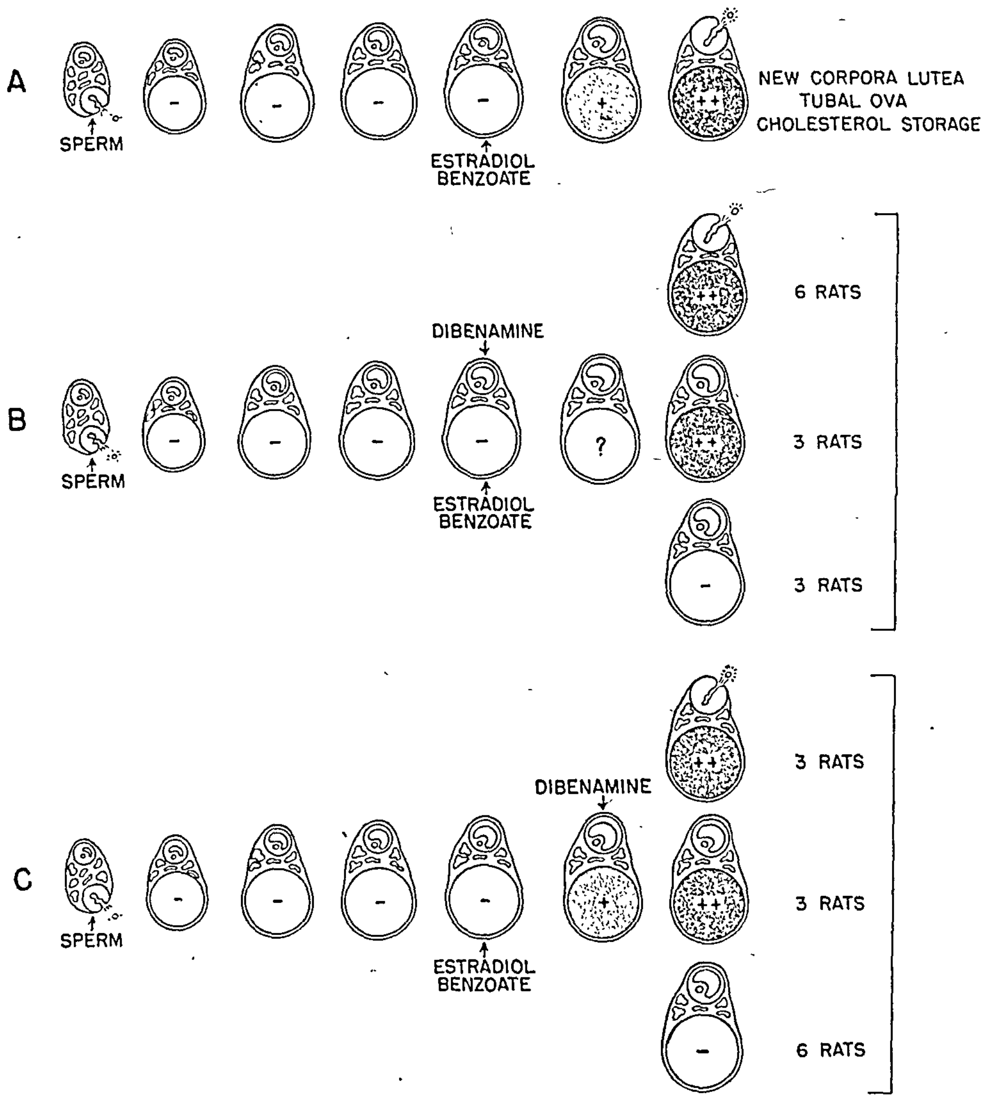


FIG. 1. Diagrammatic illustrations of experiments on the effects of estrogen and Dibenamine administration to the pregnant rat. Each horizontal group depicts the daily growth of a follicle and a corpus luteum of pregnancy from the day on which sperm were found in the vaginal smear (zero time). The symbols minus (−) and plus (+) represent degrees of luteal cholesterinization as determined by the Schultz test.

A. Estrogen, injected on the fourth day of pregnancy, induced LH release as evidenced by ovulation and storage of cholesterol in the corpus luteum of pregnancy within 48 hours. A slight cholesterinization was evident at 24 hours (Everett, 1947).

B. Dibenamine, injected just before estrogen on the fourth day, blocked estrogen-induced ovulation in 6/12 rats and blocked cholesterol storage in 3/12 rats.

C. Dibenamine, injected 20 hours after estrogen, blocked estrogen-induced ovulation in 9/12 rats and blocked cholesterinization in 6/12 rats.

hour prior to the administration of estrogen on the fourth day of pregnancy are summarized in figure 1, B. Nine rats of this series revealed strongly Schultz-positive corpora lutea and 6 of these 9 ovulated. The follicles in the 6 rats which failed to ovulate showed no evidence of stimulation by LH; they contained ova with resting nuclei and there was no indication of secondary follicular liquor.

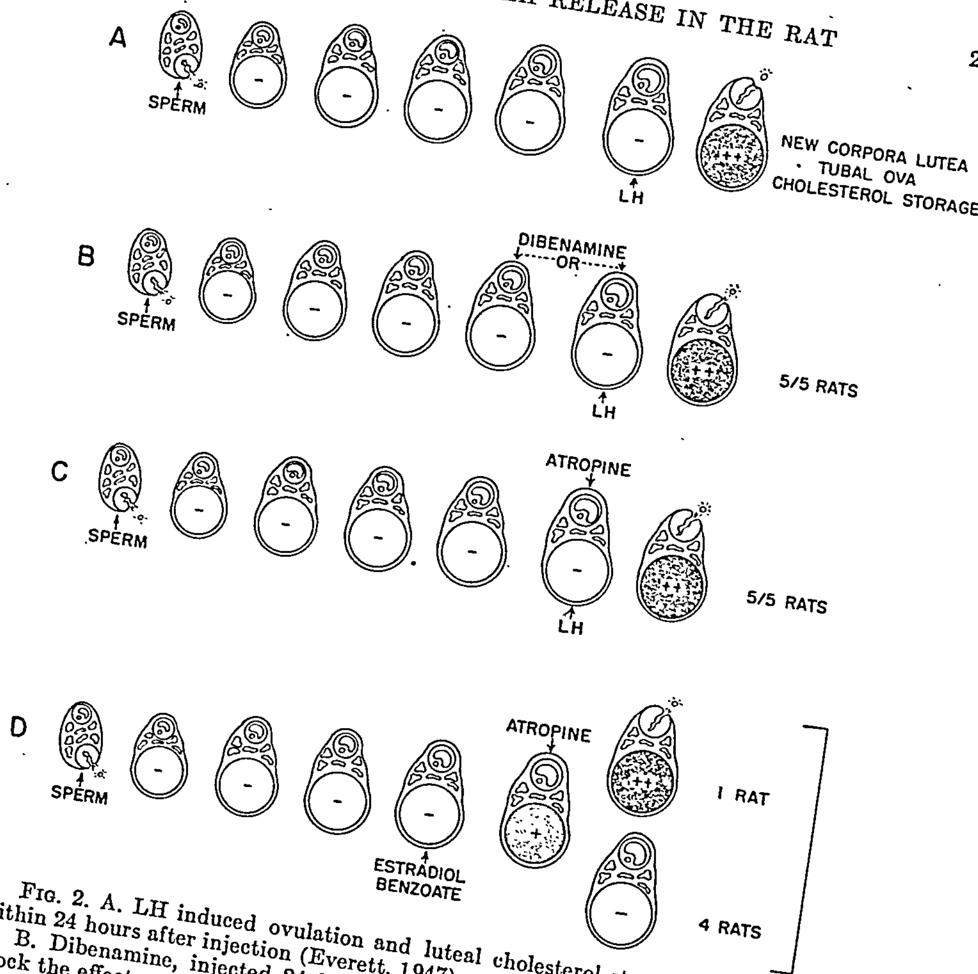


FIG. 2. A. LH induced ovulation and luteal cholesterol storage in pregnant rats within 24 hours after injection (Everett, 1947).

B. Dibenamine, injected 24 hours before or simultaneously with LH, failed to block the effects of LH on ovulation and cholesterol storage.

C. Atropine also failed to inhibit the effects of LH administration.

D. Atropine, injected subcutaneously as much as 20 hours after estradiol benzoate, quite effectively blocked the estrogen-induced release of LH as evidenced by failure of ovulation and cholesterinization in 4/5 rats.

*Injection of estrogen on the fourth day and Dibenamine on the fifth day of pregnancy.* The results of injecting estrogen at noon on the fourth day of pregnancy and Dibenamine 20 hours later are summarized in figure 1, C. Six rats stored cholesterol in their corpora lutea and 3 of these ovulated. Sections of the ovaries of the 9 rats which failed to ovulate revealed no preovulatory swelling—all of the oocytes had resting nuclei.

As was mentioned above, there was considerable positive correlation between the amount of subcutaneous mucoid deposited and the blocking effectiveness of Dibenamine. Twelve animals, among the 24 Dibenamine-treated rats, contained large amounts of mucoid. Only

2 of the 12 ovulated. On the other hand, 9 rats had little or no mucoid, and 6 of these ovulated; the other 3 had strongly Schultz-positive corpora lutea. The remaining 3 (out of 24) had a mucoid deposit of intermediate size; one was completely blocked, one stored cholesterol in corpora lutea without ovulating, and the third ovulated and had Schultz-positive (++ to +++) corpora lutea.

*Injection of LH on the fifth day and Dibenamine on the fourth or fifth day of pregnancy.* Figure 2, A graphically summarizes the results of injecting LH on the fifth day of pregnancy: ovulation and cholesterol storage in the corpora lutea of pregnancy at autopsy on the sixth day (Everett, 1947). Of 5 female rats, in the present experiments, which received LH on the fifth day, 3 received Dibenamine at 11 A. M. on the fourth day and 2 at 8 A. M. on the fifth day. As illustrated in figure 2, B, all 5 ovulated and had strongly Schultz-positive corpora lutea. The rats differed from many of the estrogen-Dibenamine-treated animals in this respect—none revealed any abdominal mucoid whatever. Whether the mucoid requires a high estrogen level for its manifestation or whether conditions attending ovulation induce its dissolution cannot be determined from the present data.

*Injection of atropine and LH on the fifth day of pregnancy.* Just as Dibenamine fails to block the effects of LH administration so does atropine. Figure 2, C summarizes the results of injecting 700 mg./kg. atropine sulfate subcutaneously at 8 A.M. and LH at 11 A.M. on the fifth day of pregnancy. At autopsy all had gravid uteri and dilated ampullae with visible ova. The ovaries revealed new corpora lutea with rupture points, a somewhat dull and watery interstitial tissue and fatty corpora lutea of pregnancy which gave a ++ to +++ Schultz reaction.

*Injection of estrogen on the fourth day and atropine on the fifth day of pregnancy.* Five pregnant rats were treated with atropine 20 hours after estrogen as illustrated in figure 2, D. Of these, 4 showed no signs whatever of stimulation by LH. The ovaries contained fatty interstitial tissue, pallid corpora lutea of pregnancy and clear, non-hyperemic follicles about 0.7 mm. in diameter. In sections these follicles were observed to contain ova with resting nuclei only. The fifth rat revealed no tubal ova nor readily visible ruptured follicles; the only external sign of stimulation was a slightly hyperemic, less fatty interstitial tissue. In sections, however, one follicle was observed to have ruptured, and another showed preovulatory changes; furthermore the corpora gave a ++ Schultz test.

Not illustrated in the diagrams are the negative results with intravenous atropine. Three pregnant rats treated with estrogen on day 4 were injected intravenously with atropine sulfate (50 mg./kg.) at 8 A.M. on day 5. At autopsy on day 6 they all showed completed ovulation, interstitial tissues considerably depleted of lipid and fatty

corpora lutea which gave strongly positive Schultz reactions. The failure of intravenous atropine to block the discharge of LH may well be due to elimination or inactivation of the drug prior to the beginning of stimulation of the LH release mechanism.

## DISCUSSION

It is a well established fact that sex hormones can in some way influence nervous activity. For instance, behavior may be considerably modified by an excess or deficiency of estrogen or testosterone (Beach, 1948). It has also long been recognized that the nervous system may be involved in the release of certain hypophyseal hormones. Luteinizing hormone in the rabbit and luteotrophic hormone in the rat are released in response to copulation or artificial neural stimulation. Furthermore, there is abundant evidence, to which reference has already been made, that estrogen stimulates or facilitates the release of luteinizing hormones in spontaneously ovulating species. To the best of our knowledge, however, our work presents the first convincing demonstration that the estrogen stimulus for the release of LH involves the intermediation of the nervous system.

Several earlier workers, notably Hohlweg and Junkman, Herold and Effkeman, and Westman and Jacobsohn, reported results which implicated the nervous system in sex-hormonal control of the hypophysis. These authors were largely concerned, however, with the production or synthesis of the gonadotrophic hormones rather than with their release. As will be pointed out below, their experiments on "release" are open to criticism.

Hohlweg and Junkman (1932) and Hohlweg (1936) observed that castration cells do not develop in hypophyses that have been removed from their normal neural relationships. They proposed a hypothetical "Sexual centrum" from which emanated the nervous impulses responsible for castration cell formation. When blood estrogen level reached a threshold value it inhibited the sex center from stimulating further production of FSH. Since, however, injected estrogen induced the same cytological changes in transplants as in intact hypophyses, they believed that estrogen's influence on LH was a direct one, independent of the nervous system. An interesting, although often overlooked, preliminary finding, mentioned by Hohlweg and Junkman (1932), is that atropine blocks the development of castration cells in the hypophyses of immature spayed rats. As was mentioned earlier, the observation that the formation of castration cells requires the integrity of the pituitary stalk, has been confirmed many times.

Herold and Effkemann (1938) reported results which purported to demonstrate that estrogen stimulates the release of LH via the nervous system. They cut the pituitary stalks in immature female rats and treated them with estrogen during the next 13 to 28 days, starting injections on the fifth to the eighth post-operative day. At autopsy

they noted the absence of luteinized ovarian follicles. Westman and Jacobsohn (1938, c), however, observed that stalk-sectioned hypophyses lose their gonadotrophic potency within 4 days. It seems probable, therefore, that Herold and Effkemann were attempting to stimulate the release of LH after the effective gonadotrophin content of the hypophysis had become depleted.

Westman and Jacobsohn (1938, b) considered neural stimuli entering along the pituitary stalk to be essential for the production but not for the release of gonadotrophic hormones. They observed that estrogen would induce luteinization of the ovaries of immature rats if injected 4 days before hypophysectomy or 2½ days before stalk section. This was interpreted to mean that the production of gonadotrophic hormone ceased when the stalk was cut but that estrogen, by a direct effect on hypophyseal cells, could liberate the LH already produced. We would suggest, rather, that the estrogen-facilitated neurogenic stimulus for the release of LH had reached the hypophysis by 2½ days and that release of the hormone involved a day and a half. In another paper Westman and Jacobsohn (1938, c) claimed to have demonstrated the stimulation of release of "gonadotrophic" hormone in adult estrous rats whose stalks had just been severed. Electrical stimulation of the vaginae, 2 to 5 hours after cutting the stalk, was followed by the development of functional corpora lutea as evidenced by deciduoma formation. Unfortunately, the experiment was not controlled to show that severing the stalk would, of itself, fail to stimulate the release of the hormone (luteotrophin in this case; it seems probable that LH had already been released since these animals were in full estrus). It should be noted that Friedgood and Bevin (1941) induced pseudopregnancy in the rat by non-specific neck operations.

There is little doubt that estrogen produces certain growth effects on hypophyseal cells independently of nervous influence, since these occur in grafts and in hypophyses whose stalks have been cut (for references see introduction). For the control of many other gonadotrophic phenomena, however, direct hormonal action has been questioned by the investigators who champion the concept of nervous intervention. The operative techniques of these workers have been criticized for damaging the hypophysis. The present work avoids this criticism by replacing surgical procedures with a chemical blocking action which does not alter the structural relationships of the hypophyseal nerves and blood vessels. The resulting complementary evidence of neural involvement in gonadal control of the hypophysis is, therefore, especially significant.

Since removal of the cervical sympathetic ganglia does not impair gonadotrophic function (Vogt, 1931, 1933) the logical pathway for transmission of nervous influence to the hypophysis is the pituitary stalk. Why, then, have so many investigators observed little or no decrement in gonadotrophic function following stalk section? It may be, as Westman, Jacobsohn and Hillarp (1943) suggest, that the

operations were incomplete and that a tuberalis-distalis connection remained intact. However, an alternative explanation is that the final pathway is the portal system and that these vessels may regenerate (Harris, 1948). Regeneration of vessels would also account for the return of function in hypophyseal transplants into the sella (Greep, 1936). Transplants to other sites have not become normally functional (Schweizer, Charipper and Haterius, 1937; Westman and Jacobsohn, 1940). May (1937), however, reported in 2 questionable cases that intraocular hypophyseal transplants in female rats were conducive to normal sex function. The fact that transplant removal did not immediately result in anestrus indicates that hypophysectomy was incomplete. Several workers who claimed that gonadotrophic function was intact after stalk section reported temporary periods of impaired function (Dempsey, 1939; Dempsey and Searles, 1943). Regeneration of portal vessels would have been prevented by Westman and Jacobsohn's (1937) operation in rats; they inserted a metal-foil barrier between the hypothalamus and the hypophysis. Westman, Jacobsohn and Hillarp (1943) emphasized the importance of the tuberalis-distalis connection. If this region was intact the whole infundibular process could be removed without interfering with sex function, but cutting the tuberalis-distalis connection led to gonadal atrophy. Westman, Jacobsohn and Hillarp favored the idea of neural control by tiny nerve fibers which they described entering the distalis through the tuberalis. Their results fit equally well the portal system hypothesis—perhaps even better, for Green (1948) has described as reticulum, fibers in the same location as the Swedish investigators' "nerve" fibers.

Humoral control of the rabbit hypophysis is supported by the results of Markee, Sawyer and Hollinshead (1946–1948). The hypophysis itself was electrically inexcitable to stimuli which, applied directly to the tuberal region of the hypothalamus, produced a maximal discharge of LH. The hormone was also liberated in response to small amounts of adrenaline injected directly into the pars distalis. The rabbit's utilization of an adrenergic agent in the natural stimulus for LH release was evidenced by the ability of Dibenamine to block copulation-induced ovulation (Sawyer, Markee and Hollinshead, 1947). Recently, atropine-block of coitus-stimulated ovulation has given indirect evidence of a cholinergic component in the neurogenic control (Sawyer, Markee and Townsend, 1949). The cholinergic component precedes, and may stimulate, the adrenergic component. Inasmuch as the estrogen-facilitated stimulus in the rat is also blocked by both adrenolytic and anticholinergic agents, the same sequence may hold for the rat. If so, then the influence of estrogen must be exerted no further peripherally than the site of action of the cholinergic component; otherwise atropine would not block it.

In the rat subcutaneous atropine blocks estrogen-induced ovulation and cholesterinization more effectively than does intravenous

Dibenamine. Dibenamine cannot be used subcutaneously because of its extreme toxicity. The reason that intravenous Dibenamine is not completely effective in blocking the stimulus for release of LH when given on the fifth day appears to be that 30 mg./kg. is not above the blocking threshold for all rats. In the fourth day injections, another factor enters in: possible partial inactivation of Dibenamine before the neurogenic stimulation has occurred. This inactivation is indicated by the fact that the same dose on the fifth day is more effective than on the fourth day. The fact that ovulation and preovulatory swelling are blocked more effectively than cholesterol storage confirms Everett's (1947) observation that in the pregnant rat, corpus luteum cholesterinization is a more sensitive indicator of LH release than is ovulation.

In what manner does estrogen operate in influenceng the neurogenic stimulation of LH release? The fact that blocking agents are still effective 20 hours after the estrogen injection, whereas certain vascular effects of estrogen are most pronounced only 6 hours after injection (Astwood, 1939) suggests that the influence of estrogen on the nervous system is a slow indirect type of action. Quite possibly the same sort of influence is exerted in this situation as in the hormonal control of mating behavior. Beach (1948) has suggested that sex hormones influence behavior most likely by altering sensitivity to external stimulation. They are not primary stimulators but they potentiate or diminish neural effects by increasing or decreasing the responsiveness of nervous mechanisms to afferent impulses. Could not estrogen in the rat influence LH release in this manner? This question is discussed at length in the following paper (Everett, Sawyer and Markee, 1949).

If extrinsic factors are primary stimuli for the release of LH in "spontaneously" ovulating species, the only fundamental difference between the rat and the rabbit, in this respect, may be the nature of the receptors and of the afferent neural pathways. It is of interest, in this connection, that estrogen appears to alter the sensitivity of the LH release mechanism in the rabbit as well as the rat. Dury and Bradbury (1943) observed that estrogen lowered the threshold to stimulateon of LH release in the rabbit by copper salts. Klein (1947) reported that copulation in the pregnant or pseudopregnant rabbit, which usually does not induce ovulation, is effective in releasing LH if the rabbit is previously treated with estrogen.

Certain methods of treatment with estrogen in the rat may so alter the sensitivity that the type of receptor mechanism, capable of inducing the release of a hypophyseal hormone, may change. The release of luteotrophic hormone (lactogen) is usually stimulated at copulation, or it may be induced by artificial vaginal stimulation. Estrogen, however, if administered at the proper time, may induce the "spontaneous" release of lactogen as evidenced by pseudopregnancy (see Sawyer and Everett, 1946, for references to the earlier literature).

Progesterone may either inhibit or facilitate release of LH in the rat, depending on dosage and time of injection (Everett, 1948). The effects of this hormone on the hypophysis may also be explained on a basis of altered sensitivity to extrinsic nervous stimulation. Just as estrogen can change the type of effective receptor mechanism so can progesterone. The persistent estrous rat will occasionally ovulate in response to mating or "spontaneously" on injection of progesterone (Everett, 1939). In the rabbit chronic treatment with progesterone inhibits the response (release of LH and ovulation) to mating (Makepeace et al. 1937) and to copper salts (Friedmann, 1941). Unpublished results of Everett and Sawyer indicate that the "stimulating" effects of progesterone in the rat are blocked by adrenolytic and anticholinergic agents.

The concept of a sex center controlling gonadotrophic activity may well be revived. In terms of the more recent evidence it would be a locus in the nervous system at which sex hormones alter the sensitivity to extrinsic stimuli and thus influence hypophyseal secretion. The "sex center" for control of mating behavoir in the cat was localized near the rostral end of the mesencephalon (Bard, 1940). In view of gonadotrophic disturbances resulting from hypothalamic lesions (Dey, 1943) and the extreme sensitivity of hypothalamic nuclei in experiments on the electrical stimulation of the release of LH (Haterius and Derbyshire, 1937; Harris, 1937; Markee, Sawyer and Hollinshead, 1946; Harris, 1948), the hypothalamus would appear to be the most likely site of the gonadotrophic sex center.

## SUMMARY AND CONCLUSIONS

It has been generally accepted that, in polyestrous animals, estrogen stimulates the release of luteinizing hormone by a direct action on adenohypophyseal cells. The present study, employing 60 mature female rats, introduces convincing evidence that the nervous system is involved in the estrogen-induced discharge of hypophyseal LH in the rat.

In the pregnant rat, injection of estrogen on day 4 is followed within 48 hours by ovulation and storage of cholesterol in the corpora lutea of pregnancy. Both of these phenomena are indicative of LH release, and they may be duplicated by injecting LH on the fifth day of pregnancy. If the potent adrenergic-blocking agent, Dibenamine, is injected prior to, or as much as 20 hours after estrogen administration, ovulation and luteal cholesterinization are prevented in a high proportion of the cases. The effects of injected LH are, however, not counteracted by Dibenamine. The anti-cholinergic drug atropine also allows injected LH to manifest its effects but, like Dibenamine, blocks estrogen-induced ovulation and luteal cholesterinization.

It has been amply demonstrated by experiments in the rabbit that Dibenamine and atropine prevent the release of luteinizing hormone only by blocking neurogenic stimuli from reaching the hypophysis.

The drugs interfere neither with the mechanism of glandular discharge of the hormone nor with the action of LH on its target organs. The present experiments demonstrate that estrogen-induced release of LH in the rat is blocked by the anti-adrenergic and anti-cholinergic drugs. It is therefore concluded that this effect of estrogen on the rat hypophysis is mediated, at least in part, by the nervous system. It is suggested that estrogen exerts its action by facilitating, or lowering the threshold to, extrinsic stimulation of a gonadotrophic sex center probably located in the hypothalamus.

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# A NEUROGENIC TIMING FACTOR IN CONTROL OF THE OVULATORY DISCHARGE OF LUTEINIZING HORMONE IN THE CYCLIC RAT[1]

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## INTRODUCTION

It was reported in the preceding paper (Sawyer, Everett and Markee, 1949) that a neural factor is involved in the release of LH[2] from the female rat hypophysis in response to estrogen treatment, thus extending to a "spontaneously" ovulating species principles which had been previously disclosed in the rabbit (Markee, et al., 1946, 1948; Sawyer, et al., 1947, 1948). Although estradiol benzoate injected on the fourth day of pregnancy in the rat regularly induces ovulation within 48 hours (Everett, 1947), this effect could be prevented in most cases by Dibenamine or atropine, administered as late as 20 hours after the estrogen. It was proposed that estrogen modifies the threshold of activation of an hypothalamic center, or of certain afferent pathways leading to the "center," and that a chief difference between the rabbit and rat lies in the particular afferent pathways concerned.

While in the domestic rabbit the most important nervous pathways concerned with the LH-release mechanism are those which are activated directly or indirectly by copulation, these are generally thought to have no influence on discharge of luteinizing hormone in the rat. Here, the only exteroceptive pathways known to be concerned in regulation of the onset of estrus and of ovulation time are optic (Hemmingsen and Krarup, 1937; Browman, 1937) and perhaps thermal (Lee, 1926; Browman, 1943, b; Dempsey and Searles, 1943). In the absence of light the normal nocturnal activity and estrous rhythms can be maintained or reversed by maintaining or reversing the 24-hour rhythm of temperature fluctuation (Browman, loc. cit.). Conceivably, as estrogen secretion increases, the thresholds in these

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[2] The expression LH is used in this paper in the conventional sense (cf. Hisaw, 1947), recognizing that FSH also enters into preovulatory stimulation of follicles. The effects with which we are concerned here may largely express quantitative rather than qualitative differences in gonadotrophin secretion.

systems and in a variety of others are lowered (Beach, 1948), eventually resulting in a discharge from the "sex center" in the hypothalamus.

It was obviously important to extend the experiments to the normal cyclic ovulation. The results of that study are the subject matter of the present report. It will be demonstrated that cyclic ovulation can be blocked by injection of either Dibenamine or atropine as late as 2 P.M. on the day of proestrus. Dibenamine gives somewhat less constant results than atropine. Injections of either drug at 4 P.M. are almost entirely ineffective, indicating that in our colony neurohumoral stimulation of the anterior lobe occurs sometime between 2 and 4 P.M., about 9 to 11 hours before ovulation. A preliminary account of a part of these results was recently published (Everett, Sawyer and Markee, 1948).

## MATERIALS AND METHODS

The techniques of administration of Dibenamine and of atropine were those described by Sawyer, Everett and Markee (1949). Dibenamine was injected intravenously, the concentration being 10 mg./ml. and the standard dose 30 mg./kg. Atropine sulphate at a concentration of 70 mg./ml. in Ringer-Locke solution was injected subcutaneously, the standard dose being 700 mg./kg.

In this study 87 cyclic female rats of the inbred Vanderbilt (Osborne-Mendel) strain were used. The definitive experimental groups, comprising 68 rats, include only animals having histories of regular 4-day cycles (substrain Va). The remaining 19, used during the exploratory stage of the work, were 5-day cyclic females mostly of the substrain Vc. These two substrains have recently been described (Everett, 1948) with respect to predictability of length of cycle and of the time of ovulation. It was stated that in the Va substrain, in which most cycles are 4 days long, if a given animal has experienced 2 observed 4-day cycles in sequence the probability is about 93% that the next cycle will also be of that length. In the Vc substrain, on the other hand, most females are 5-day cyclic. After 2 successive observed cycles of that duration the probabilities are about 90% that the next cycle will be similar, 94% that it will not be prolonged. These probabilities are further improved by selection of animals showing characteristic proestrous vaginal smears on the morning of expected proestrus (8–10 A.M.). In the 4-day cyclic group 25 to 30% of the animals still present a few leucocytes in the smears at this time. Where such rats were used the degree of vaginal intumescence was employed as an additional criterion and an early afternoon smear was prepared in confirmation. No attempt was made to use the criterion of psychic estrus, as the systemic effects of the drugs are such that no reliance could be placed upon it. The time of ovulation in the 4-day Va rats is between 1 and 2:30 A.M. during the early morning of the day following proestrus. Comparable data on 5-day cyclic rats have not been obtained, but the histological appearance of their new corpora lutea during the late morning after ovulation (vaginal stage III) strongly indicates that here also ovulation occurs not long after midnight.

The regularity of cycles and the constantly close relationship of ovulation

time to hours of the day may be ascribed to the genetic homogeneity of the animals and to the uniformity of the diurnal rhythm of lighting. The colony lighting is entirely controlled by a time switch, the lights being on for 14 hours each day. The period of darkness is thus 10 hours.

The experimental procedure in general outline was as follows: One or the other drug was administered at a certain hour of the day of proestrus (8–9 A.M., 2 P.M., 4 P.M., 5 P.M., or 6:45 P.M.). Autopsy was routinely performed on the following morning between 8 and 10:30 A.M., after killing the animal with illuminating gas. (In a few cases, as specified later, the experiments were continued longer.) The status of the uteri was noted with respect to hyperemia, reactivity to manipulation and, especially, degree of distention and fluid content. Both ovaries with tubes attached were removed to a dish of physiological saline. Under a dissecting microscope the presence or absence of dilatation of the ampullae of the oviducts was observed. The ampullae were excised and transferred to a drop of saline on a microscope slide. After straightening their coils, a cover slip was applied and the preparation was observed under the low power of a compound microscope by transmitted light. Tubal ova, if present, are usually readily visible by this means. Each ovary was then carefully freed from surrounding structures by incising the capsule and clipping the mesovarium with iridectomy scissors. The status of follicles was observed—whether clear or hyperemic, degree of hyperemia, approximate diameter, etc. Rupture points were looked for. The appearance of the interstitial tissue was recorded—whether markedly fatty as it is normally during proestrus, or dull and watery, indicative of the cholesterol depletion which takes place during estrus (see below). The corpora lutea of the preceding cycle were observed for the apparent degree of fat content. On the day after ovulation they are normally distinctly creamy, in contrast with a relatively pallid aspect during proestrus (Everett, 1945).

Routinely, one or both ovaries were preserved in Zenker's fluid for paraffin imbedding and serial sectioning. Where a full set of tubal ova was found (6 to 11), such preparations often seemed unnecessary and in only a few such instances did we serially section even one ovary. In representative cases in most of the experimental groups portions of one or both ovaries, or an entire ovary, were fixed in 10% formalin. Frozen sections were cut at 30$\mu$ thickness and were examined by the Schultz test for cholesterol content of the interstitial tissue and corpora lutea (Everett, 1945, 1947). One index of LH action on the ovary is depletion of interstitial cholesterol content (Claesson and Hillarp, 1947), tending to be most pronounced near ripe follicles and in the vicinity of new corpora lutea (Everett, 1947 and unpublished). Increased cholesterol storage in corpora lutea of the previous cycle is also a measure of LH action under certain conditions (Everett, 1945, 1947). Thus, our 3 criteria of LH secretion are: (1) ovulation or preovulatory swelling; (2) cholesterol depletion in the interstitium, as evidenced grossly by a dull, watery appearance or microscopically by a negative or very weak Schultz test; (3) cholesterol storage in the corpora lutea of the preceding cycle, as evidenced by a strongly positive Schultz reaction or simply a "creamy" appearance at the time of autopsy.

## RESULTS

### *Dibenamine*

Since in pregnant rats treated with estrogen, Dibenamine had been successful in preventing ovulation when injected at 8 A.M. on the morning before expected ovulation (Sawyer, Everett and Markee, 1949), this hour on the day of proestrus was chosen for the preliminary trials in cyclic rats. Of the 10 animals in this initial experiment 6 were 5-day cyclic Vc rats and 4 were 4-day cyclic Va rats, all having proestrous smears at injection time (8 to 9:30 A.M.). At autopsy on the following morning none had tubal ova. No rupture points were visible under the dissecting microscope. With the exception of 2 of the 4-day rats, histological study of the ovaries revealed no evidence of preovulatory swelling of the follicles. In the 2 exceptional cases a few follicles were found to be in early stages of swelling with first polar body divisions in progress or complete. Since the control experiments with LH in the pregnant rat (Sawyer, Everett and Markee, loc. cit.) had shown that Dibenamine does not interfere with the action of LH on the ovary, the present results left no doubt that adrenergic stimulation of the hypophysis occurs during the day of proestrus and that this stimulation is essential for secretion of LH sufficient to induce ovulation. By treatment at various hours of the day it should thus be possible to determine time limits within which the stimulation occurs.

Because of the greater availability of Va stock and the more complete information about ovulation time in the 4-day cycle we subsequently confined our attention principally to 4-day cyclic animals. The 8–9 A.M. injection group was increased by 12 rats (9 autopsied at the usual time and 3 autopsied about 48 hours after injection). Thus a total of 16 4-day cyclic rats was treated between these hours. In table 1 are listed the 13 cases which were autopsied 24 hours later. Ovulation was blocked in 9 cases, but the presence of preovulatory swelling in a few follicles in 2 instances (mentioned in the preceding paragraph) indicated a slight amount of LH secretion. Two rats had 2 and 3 tubal ova, respectively. In one of these rats, sections of both ovaries contained 2 rupture points, 2 follicles in early swelling and 5 unstimulated follicles with resting ovocyte nuclei. In the other in which the ova were confined to the right tube, sections of the left ovary contained 3 "unstimulated" follicles and none which had advanced beyond that stage. These 2 cases, therefore, also indicate less than the normal LH secretion. The other 2 rats were judged to have fully ovulated, although one had only 5 tubal ova, 4 on the right and 1 on the left. Her left ovary contained only 1 ruptured follicle, but of 4 follicles with resting ovocyte nuclei, 2 exhibited localized theca luteinization. It is possible that this case also represents diminished LH secretion resulting from the treatment, but the uncertainty led

to its classification as complete ovulation. The other rat had 8 tubal ova and her reproductive tract otherwise gave a completely normal picture of late estrus.

Of the 3 rats which were autopsied 48 hours after the 8 A.M. injection, 2 failed to ovulate and 1 had a set of new corpora lutea. One

TABLE 1. SUMMARY OF DATA SHOWING STATUS OF FOLLICLES AND EXTENT OF OVULATION ON THE MORNING AFTER TREATMENT WITH DIBENAMINE OR ATROPINE

*DIBENAMINE*

| Hour injected | No. of rats | Tubal ova | | | Follicles | | | Ovulation | |
|---|---|---|---|---|---|---|---|---|---|
| | | None | Few | Many | No swelling | Early swelling | Late swelling | In progress | Complete |
| | | 9 | | | 7 | 2 | | | |
| 8–9 A.M. | 13 | | 3 | | | | | 2 | 1 |
| | | | | 1 | | | | | 1 |
| | | 12 | | | 7 | 2 | 2 | 1 | |
| 2 P.M. | 19 | | 4 | | | | | 4 | |
| | | | | [3][1] | | | | | [3][1] |
| | | 3 | | | | 1 | 1 | 1 | |
| 4 P.M. | 10 | | 1 | | | | | | 1 |
| | | | | 6 | | | | | 6 |
| | | 1[2] | | | | | 1[2] | | |
| 5 P.M. | 4 | | | | | | | | |
| | | | | 3 | | | | | 3 |
| 6:45 P.M. | 4 | | | 4 | | | | | 4 |

*ATROPINE*

| Hour injected | No. of rats | Tubal ova | | | Follicles | | | Ovulation | |
|---|---|---|---|---|---|---|---|---|---|
| | | None | Few | Many | No swelling | Early swelling | Late swelling | In progress | Complete |
| 8 A.M. | 5 | 5 | | | 5 | | | | |
| 2 P.M. | 5 | 5 | | | 5 | | | | |
| | | | 1 | | | | | | 1 |
| 4 P.M. | 5 | | | | | | | | |
| | | | | 4 | | | | | 4 |

[1] Autopsied at 4 P.M., ca. 26 hours after injection.
[2] Found dead but still warm at 7·45 A.M.

ovary of each rat was serially sectioned, disclosing in the anovulatory cases "unstimulated" follicles, nearly all of which were atretic. The new corpora in the third animal were somewhat less advanced than normal for this stage of the cycle. Ovulation had probably been retarded. The vaginal smears in all 3 rats were metestrous, a marked invasion of leucocytes being seen in all. Thus, the interruption of estrogen secretion is indicated, a matter to be discussed later.

As shown in table 1, 19 4-day cyclic rats were injected at about 2

P.M. on the day of proestrus. Nine of them were autopsied between 8 and 9 A.M. the next day, 1 at 12:45 P.M. and 9 at about 4 P.M. Tubal ova were absent in 5 of the first 9, in the 12:45 case and in 6 of the 9 killed in late afternoon. Seven of these 12 animals gave no indication of preovulatory swelling. Three (among the morning autopsy series) exhibited preovulatory swelling in variable degree. The rat autopsied at 12:45 had 3 very recently ruptured follicles which would undoubtedly have been in late swelling if she been killed as early as the others; she is so classified in table 1. One rat in the morning group had 5 follicles in late swelling and a single ruptured follicle, but the ovum had apparently not yet reached the tube. Four others in the morning group had 1 or 2 tubal ova and histological study of their ovaries disclosed from 1 to 4 ruptured follicles and a variety of stages of preovulatory swelling. Three of the rats killed at 4 P.M. had completely ovulated (10 to 11 ova). Although they are classified in table 1 as complete ovulations, they would not necessarily have been so had they been autopsied at the morning hour. In one of them tertiary follicular fluid had not yet been formed in large amount, strongly suggesting delayed ovulation.

The group injected at 4 P.M. comprises 10 rats (table 1), all of which were autopsied the following day between 8 and 9 A.M. In none of these was LH release completely blocked, although tubal ova were absent in 3 animals. All 3 had follicles in preovulatory swelling. In one of them, 2 follicles had already ruptured and in another all 10 follicles were very near the time of rupture. A fourth rat had 5 tubal ova and 7 rupture points. The remaining 6 rats had from 7 to 10 tubal ova and full sets of new corpora lutea which were histologically identical with those normally found at this time in 4-day cyclic rats.

The results of injection at 5 and 6:45 P.M. may be considered together. Four rats were injected at each of these hours. One animal in the 5 o'clock group was found dead but still warm shortly before 8 A.M. the next morning. She was autopsied immediately. No tubal ova were found, nor ruptured follicles, but histological study of one of the ovaries disclosed 5 follicles in late preovulatory swelling. The vaginal smear was still in late proestrus. It is possible that the retardation of follicle maturation and of the vaginal changes was due to non-specific systemic factors associated with the moribund condition during the night. The other 7 rats had fully ovulated, having from 6 to 10 tubal ova.

In the experiments with pregnant rats treated with estrogen (Sawyer, Everett and Markee, 1949), the occurrence of subcutaneous "mucoid" as a sequel to Dibenamine treatment was closely correlated with the blocking effect of this agent. In the present experiments, however, such correlation was less complete. In 4-day cyclic rats of the 8–9 A.M. and 2 P.M. injection groups, among 26 cases in which our records include reference to mucoid and in which ovulation was either

completely or partially blocked, 17 rats had mucoid at autopsy while 9 had none. Its presence or absence does not, therefore, appear to be an acceptable criterion of adequacy of Dibenamine dosage under these conditions.

*Atropine*

It was apparent from the results with Dibenamine that injections at 2 P.M. or earlier usually result in either complete or partial block of LH release, while injections at 4 P.M. or later usually do not interfere with the release mechanism at least so far as ovulation is concerned. Evidently adrenergic stimulation of the hypophysis occurs mainly in the interval between 2 and 4 P.M. in 4-day cyclic rats in this colony. Since a cholinergic link in neurogenic stimulation of LH release has been indicated in the rabbit (Sawyer, Markee and Townsend, 1949) it was of interest to determine whether a cholinergic link also occurs in the cyclic rat. Concurrent experiments with pregnant rats (Sawyer, Everett and Markee, 1949) demonstrated that ovulation after estrogen administration is prevented by atropine injected on the day before ovulation is expected. Yet atropine, like Dibenamine, does not prevent ovulation in response to injected LH.

Five 4-day cyclic rats were injected with atropine at 8 A.M. On the following morning the vaginal smears were still proestrous. At autopsy about 24 hours after injection the reproductive tracts were uniformly characteristic of a proestrous rat. No tubal ova were to be seen (table 1). The follicles were large, but not hyperemic. In histological section no evidence of normal preovulatory swelling was seen. In one rat a single follicle among 9 showed the first maturation division in progress, but as mitotic divisions could not be found in the granulosa this follicle was judged to be atretic. In a second rat a single follicle among 9 was atypical. The status of the ovocyte nucleus could not be determined because of a few missing sections, but there was no secretion of secondary liquor about the cumulus. The granulosa was compact and of irregular thickness. Its cells were disoriented as in late preovulatory swelling and mitotic figures were most unusually numerous. The basement membrane was intact. Whether this follicle represents slight stimulation we cannot state. All others (7–8 in each of the 5 rats) appeared to be normal, growing follicles with resting ovocyte nuclei.

It was apparent from these results that a cholinergic link exists in the LH-release mechanism of the cyclic rat. To estimate the time limits within which this part of the mechanism operates, atropine was injected into five 4-day cyclic rats at 2 P.M. on the day of proestrus and into 5 others at 4 P.M. (table 1). The animals were killed and autopsied the following day between 8 and 9:15 A.M. In the 2 o'clock group not one rat had tubal ova. Their ovaries presented the characteristic aspect of ovaries seen in normal proestrus, with large clear follicles. Histological study showed these to be healthy, growing follicles with

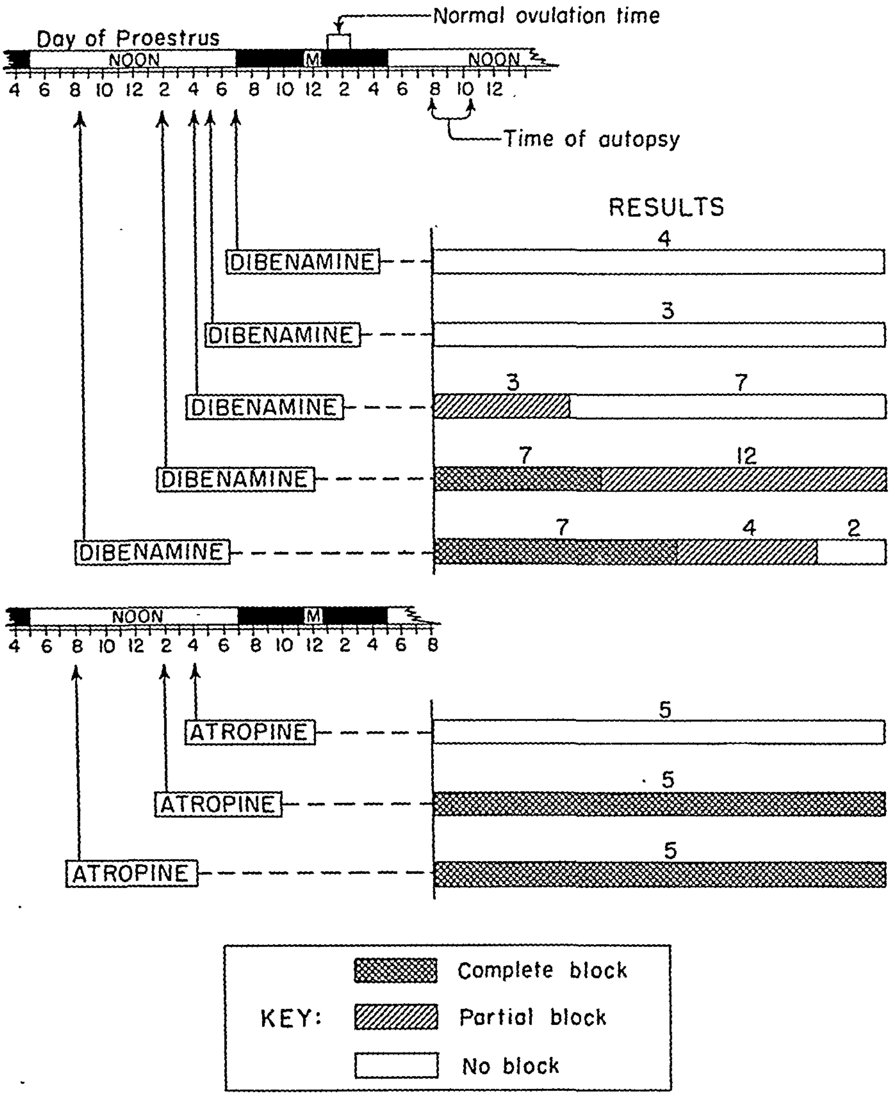


FIG. 1. Graphic representation of the degrees of blocking of the LH-release mechanism obtained by injecting Dibenamine or atropine at various hours of the day of proestrus, in 4-day cyclic rats. The numbers over the various segments of the bars under "RESULTS" indicate the respective numbers of rats represented by the proportional lengths of the segments.

resting ovocyte nuclei in all instances. In the 4 o'clock injection group the results were quite different (table 1). Four of the 5 rats had full sets of tubal ova (7–10). The fifth rat had 3 observed tubal ova, 1 on the right and 2 on the left. Her right ampulla, however, was exceedingly dilated and the right ovary when examined histologically was found to contain 5 new corpora lutea in a comparable stage of development with those in the other 4 rats.

In figure 1 the above data from the Dibenamine and atropine se-

ries are summarized in graphic form, with respect to the apparent degree of block of the LH-release mechanism. With Dibenamine, partial or complete block is the rule when injections are made at 2 P.M., or earlier, on the day of proestrus. With atropine, complete block is the rule under like conditions. With Dibenamine, ovulation usually proceeds unhampered following injections at 4 P.M. or later. The same is true with atropine. No real difference can be claimed between the 4 P.M. Dibenamine series and the 4 P.M. atropine series, on the basis of the present numbers of cases.

TABLE 2. CORRELATION BETWEEN VAGINAL SMEAR RETARDATION AND EXTENT OF BLOCKING BY DIBENAMINE

| Vaginal smear stage (morning of autopsy) | Hours of Dibenamine injection (preceding day) | Degree of blocking, measured by ovulation | | |
|---|---|---|---|---|
| | | Blocked (no. of rats) | Partially blocked (no. of rats) | No block (no. of rats) |
| Late proestrus | 8–9 A.M. | 1 | 3 | 1(?)[1] |
| | 2 P.M. | 5 | 7 | |
| | 4 P.M. | | 4 | |
| | 5 P.M. | | 1[2] | |
| | All hours | 21 | | 1 |
| Estrus | 8–9 A.M. | 8 | 2 | 1 |
| | 2 P.M. | 2 | 2 | |
| | 4 P.M. | | | 6 |
| | 5 P.M. | | | 3 |
| | 6:45 P.M. | | | 4 |
| | All hours | 14 | | 14 |

[1] 5 Tubal ova, 2 follicles partly luteinized but not ruptured.
[2] Found dead but still warm at 7:45 A.M.

*Correlation between status of ovaries and status of vagina and uterus.* In the atropine series there was a clear-cut correlation between incidence of full ovulation and of cornified vaginal smears. All rats injected with atropine at 2 P.M. or earlier had proestrous smears the next morning. The one animal in the 4 P.M. injection group which had a reduced number of tubal ova at autopsy time, also had a late proestrous smear. The others were fully cornified.

In the Dibenamine series, the correlation was somewhat similar but less clear-cut (table 2).[3] The principal lack of conformity was in the 8–9 A.M. injection group where 8 of 9 anovulatory rats,[4] showing no preovulatory swelling, were nevertheless fully cornified 24 hours after injection. Considering all Dibenamine groups together, the incidence of proestrous smears was greatest in the animals showing partial block, strangely enough. Among the "blocked" and "partially

[3] The cases admitted to tables 2 and 3 are not in all instances those recorded in table 1. For various reasons a few of the latter are excluded and certain others are pertinent.
[4] Including 2 rats autopsied at 48 hours.

blocked" groups combined, 21 of 35 rats had proestrous smears on the morning after injection. On the other hand, 14 of 15 rats in which ovulation was not significantly delayed were fully cornified.

As regards the uteri in the Dibenamine series, a similar correlation was found (table 3)[3] between uterine distention and the prevention or retardation of follicle maturation. Thus, among 24 "blocked" or "partially blocked" rats, 19 exhibited some degree of uterine distention, while this was seen in only 4 out of 15 of the "not blocked" group. In the atropine series all of the 8 o'clock injection group and 4 of the 2 o'clock group had distended uteri. In the 4 o'clock group 2 rats had slightly distended uteri, including the animal in which the vaginal smear was proestrous. In the other 3 no distention was seen.

TABLE 3. CORRELATION BETWEEN UTERINE DISTENTION AT AUTOPSY ON THE MORNING AFTER DIBENAMINE INJECTION, AND EXTENT OF BLOCKING

| Status of uteri | Hour of Dibenamine injection (preceding day) | Degree of blocking, measured by ovulation | | |
|---|---|---|---|---|
| | | Blocked (no. of rats) | Partially blocked (no. of rats) | No block (no. of rats) |
| Distended (proestrous) | 8–9 A.M. | 5 | 3 | 1(?)[1] |
| | 2 P.M. | 1 | 8 | |
| | 4 P.M. | | 2 | 2 |
| | 6:45 P.M. | | | 1 |
| | All | 6 | 13 | 4 |
| Not distended (estrous) | 8–9 A.M. | 1 | 1 | 1 |
| | 2 P.M. | | 1 | |
| | 4 P.M. | | 2 | 4 |
| | 5 P.M. | | | 3 |
| | 6:45 P.M. | | | 3 |
| | All | 1 | 4 | 11 |

[1] 5 Tubal ova, 2 follicles partly luteinized but not ruptured.

*Status of interstitial tissue and corpora lutea of the preceding cycle.* There was a close correlation between the degree of follicle maturation or ovulation and the degree of depletion of interstitial lipid. In the Dibenamine group, all "blocked" rats had a fatty interstitium. Fifteen of 20 "partially blocked" rats were similar, while 5 showed at least local depletion near ruptured and preovulatory follicles. Among 15 rats which were "not blocked," interstitial lipid was partially or strongly depleted in 14 cases. The atropine series gave similar results.

Cholesterol storage in the corpora lutea of the preceding cycle was surprisingly rare, even in the animals injected at 4 P.M. or later, in both the Dibenamine and the atropine series. Schultz-positive corpora lutea were found in only 3 Dibenamine-treated rats in the definitive (4-day cyclic, 24-hour autopsy) series: 1 of the completely ovulated rats of the 8–9 A.M. injection group, 2 completely ovulated rats of the

4 P.M. group. The corpora were uniformly Schultz-negative in the 7 rats which ovulated in spite of injection of Dibenamine at 5 to 7 P.M. Where autopsy time was delayed until late afternoon, 26 hours after 8 A.M. Dibenamine injection, or longer (48 hours), 3 of the 4 rats which ovulated had distinctly fatty corpora lutea. Schultz tests were not performed. In the atropine series the corpora were grossly pallid in all cases. Schultz tests were performed on corpora from 3 of the 4 o'clock injection group and from one each of the other groups. All were negative.

## DISCUSSION

Before considering the broad implications of the above experiments certain details require evaluation. While atropine proved to be completely effective in blocking ovulation when injected at 8 A.M. or 2 P.M., Dibenamine completely blocked only 14 of the 32 4-day cyclic rats at these hours. These facts might be construed to mean that in the rat the adrenergic component of the LH-release mechanism precedes the cholinergic. This interpretation is contradicted, however, by the 4 P.M. data and by unpublished data based on the phenomenon of advancement of ovulation time by progesterone. When progesterone is administered on the third day of diestrus in 5-day cycles, ovulation time is advanced about 24 hours (Everett, 1944, a; 1948). Injection of Dibenamine just after progesterone, or 4 hours before, completely blocked this effect in a number of cases, but in several instances a few follicles were found in preovulatory swelling (on the morning of normally expected proestrus).

The present evidence indicates, then, that Dibenamine in the cyclic rat, as in the pregnant, estrogen-treated rat (Sawyer, Everett and Markee, 1949), is not a perfect blocking agent. The partial effects observed in cyclic rats were, however, expressed somewhat differently. Where in the pregnant rats subovulatory LH activity was detected by cholesterinization of the pregnancy corpora lutea, in cyclic rats diminished LH secretion was made evident chiefly by retardation of follicle maturation. The degree of lipid depletion in the interstitial tissue proved, in the cyclic rat, to be a better indicator of LH than corpus luteum cholesterinization. The general absence of luteal cholesterol storage on the morning after Dibenamine or atropine treatment, even when full ovulation occurred, may, of course, indicate moderate lowering of LH secretion. It is significant that in 3 of the 4 ovulated rats, in which autopsy was delayed considerably beyond the usual time after Dibenamine treatment, the corpora lutea of the previous cycle were fatty. It thus seems probable that many of the negative cases in the definitive series would have become Schultz-positive a few hours later. More information is needed about the respective time-response relationships of ovulation, interstitial cholesterol depletion and luteal cholesterinization to various quantities of LH.

Considering the 8–9 A.M. injection group as a measure of the in-

complete blocking effects of Dibenamine and thus a control for the 2 P.M. group, the Dibenamine and atropine series are in close agreement. They demonstrate that in the particular strain of rats with which we worked, during proestrus of the 4-day cycle, a neurogenic stimulus passes to the hypophysis between 2 and 4 P.M. in most animals. This stimulus has both cholinergic and adrenergic components. The evidence from the rabbit (Sawyer, Markee and Townsend, 1949) would indicate that the cholinergic component precedes the adrenergic. We can only assume that the same relationship is true in the rat. We must also draw on the rabbit studies for the most crucial evidence that neither Dibenamine nor atropine impairs the ability of the hypophysis to liberate LH. If injection of either agent in rabbits is delayed for even a minute after coitus, ovulation proceeds without interference. Hence LH liberation *per se* is not affected, for that process is known to require about an hour in the rabbit (Fee and Parkes, 1929).

The fact that the majority of rats in which ovulation was delayed or completely prevented also experienced retardation of the vaginal smear sequence is of great importance in itself. This inhibition of vaginal smear changes in conjunction with blocked ovulation constitutes the reverse of conditions described in 5-day cyclic rats in which ovulation time was advanced 24 hours (Everett, 1944, a; 1948) by progesterone therapy. Under those conditions the vaginal sequence was accelerated; here it is retarded. The significance of these effects has been discussed in the recent report of the progesterone experiments (1948).

The demonstration of a chronologically limited neurogenic stimulus for the ovulatory discharge of LH from the adenohypophysis, for the first time indicates a means by which at least one of the chronologic features of the polyestrous cycle may be synchronized with environmental rhythms. Synchronization of the cyclic activity of the hypophysis-gonad system with environmental factors is a well recognized fact in many species of mammals and lower vertebrates (Marshall, 1936, 1942; Bissonnette, 1936; Beach, 1948). Such effects are best seen in the seasonal breeders, in several of which it has been well established that conditions of illumination are of paramount importance. Among constant breeders the effects of environment are generally more obscure. Among polyestrous, spontaneously ovulating species it has usually been thought that the rhythmic interplay between hypophysis and ovary proceeds as a humoral mechanism largely without neural intervention.

Nevertheless, there are clear indications that the cycles of rats, mice, guinea pigs and hamsters are synchronized with diurnal rhythms of illumination and temperature. Thus it has been well recognized that rats usually ovulate and exhibit maximal estrus during the dark period. As a corollary of this fact, Hemmingsen and Krarup state: (1937, p. 28) "The length of an oestrous cycle must always be a whole number of days." These authors were able to reverse the nocturnal

rhythms of running activity and of maximal estrus by keeping rats in darkness during the solar day and illuminating them at night. Similar effects have been described in the mouse (Snell, et al., 1940; Gresson, 1940). Snell, et al. reported, furthermore, that in mice under such reversed lighting, ovulation usually occurs between the artificial "midnight" and 3 "A.M." It has been stated that ovulation in the golden hamster occurs quite precisely between midnight and 2 A.M. (Ward, 1946). The guinea pig has been reported to exhibit maximal estrous behavior during the night (Young, Myers and Dempsey, 1933).

In our inbred strain of rats under regulated conditions of illumination, animals which are carefully selected for regularity of cycles ovulate within the predictable time limits of 1:00 to 2:30 A.M. The neurogenic stimulus to the hypophysis reaches the gland, as we have now shown, between 2 P.M. and 4 P.M. of the preceding afternoon, roughly 10 to 12 hours before ovulation. This interval is remarkably similar to that between copulation and ovulation in the rabbit (Fee and Parkes, 1929; Waterman, 1943, and refs. cited there). In the rat the actual hours reported here for both stimulation and ovulation may well vary in other strains and under other conditions. In colonies in which lighting is not controlled a considerable irregularity may occur and where periods of controlled lighting per day are other than 14 hours deviations may be expected from the time limits which we observed.

In the preceding article of this series (Sawyer, Everett and Markee, 1949) it was proposed that the concept of a "sexual center" in the hypothalamus be revived. The present evidence gives additional support for this view and allows us to speculate upon some of the functional characteristics of such a center in the rat, under different hormonal and environmental conditions.

A principal factor leading to discharge of the "ovulation stimulus" from the "center" is probably an elevated estrogen level *per se* or some metabolic condition associated with elevated estrogen. This may act directly in the hypothalamus or may modify the afferent impulses feeding into it (Beach, 1948). Progesterone may synergize importantly with estrogen in this action, for under certain conditions progesterone facilitates ovulation in this species (Everett, 1948). It is noteworthy that Kent, (1948) reports that castrated, estrogen-primed hamsters are brought into psychic estrus as early as 20 minutes after introduction of a minute amount of progesterone into the cerebral ventricles.

The rhythm of daily illumination is concerned in the rhythmicity of the "center." This effect does not appear to be a direct one, however, for when rats are placed in nearly constant darkness they remain regularly cyclic (Browman, 1937–1943), even when temperatures are

constant. In fact, they often retain preceding nocturnal rhythms of activity and estrous behavior. Such effects of illumination are mediated by the optic nerve (Browman, loc. cit.). Subservient to rhythms of light and darkness, diurnal rhythms of temperature are also important in conditioning the activity and estrous rhythms (Browman, 1943, b). This becomes apparent during constant darkness or after enucleation or cutting the optic nerve.

Certain effects of lighting may be quite immediate. Exposure of rats to continuous light often induces the persistence of follicles and of vaginal estrus (Browman, 1937–1943; Hemmingsen and Krarup, 1937). In animals which are especially susceptible these effects may begin during the estrus which immediately follows the introduction of continuous light (Everett, 1942 and unpublished). Such responses to continuous light also depend on intact optic nerves (Browman, loc. cit.) and probably to a large extent upon the hormonal status. Progesterone facilitates the cyclic action of the LH-release mechanism during continuous light (Everett, 1940, a). Indications are that in "light-susceptible" rats the hypothalamic mechanism for the stimulation of LH-release is refractory to estrogen, perhaps because of faulty progesterone secretion (Everett, 1942–1948).

Other effects of lighting are delayed in their expression. Reversal of estrus by reversed day-night requires about 10 days (Hemmingsen and Krarup, 1937). A certain strain of rats (DA) in our colony tends to become anestrous after the daily light ration is reduced to less than 10 hours. This effect is usually first indicated, by slightly prolonged diestrous intervals, about 15 days after the new conditions are established. The full effect may not be registered until 30 days or later (Everett, 1942 and unpublished). Another curious effect of short day-length in this strain is observed in older females which have become "spontaneously" persistent-estrous during 14-hour days. Two weeks to a month after the establishment of "short days," cycles often reappear. These cycles are accompanied by renewed corpus-luteum formation (unpublished).

In the phenomenon of persistent estrus, either "spontaneous" or light-induced, we are dealing with a condition which is not uncommon in older females of many species of mammals (follicle retention cysts with chronic estrogen secretion). It would seem that one direct cause of such aberrant occurrences is the failure of the hypothalamus to discharge an adequate stimulus to the hypophysis. As a result the hypophysis continues more or less steadily to secrete only small amounts of gonadotrophin which are nevertheless sufficient to maintain estrogen secretion. This steady state is normal in such species as the rabbit, but subject there to interruption by the imposition of a set of afferent neural impulses associated with copulation. Significantly the steady state of persistent estrus in the rat may similarly be

interrupted by imposition of new conditions: (1) occasionally by copulation (Everett, 1939 and unpublished); (2) by shortened daylength as mentioned above; (3) by progesterone therapy (Everett, 1940, b; 1943); and (4) by daily treatment with desoxycorticosterone or with small doses of testosterone propionate (Marvin, 1947, 1948).

Whether secretion of gonadotrophin during such steady states requires neurogenic stimulation remains uncertain. It has been reported that moderate secretion can occur in hypophyses transplanted to the kidney or anterior chamber of the eye (Martins, 1936; Schweitzer, Charipper and Haterius, 1937). However, Westman and Jacobsohn hold to the view that the central nervous system maintains a steady trophic action on the hypophysis which is essential for adequate synthesis of gonadotrophin (see references and discussion in Sawyer, Everett and Markee, 1949). With regard to this question it is perhaps significant that in 3 of the present experiments, in which autopsy was delayed until 48 hours after 8 A M. Dibenamine injection, the vaginal smears became leucocytic not only in the rat which ovulated but in the other two as well. In the latter, marked follicular atresia was found. It would appear that in these two instances Dibenamine not only prevented the ovulatory discharge of gonadotrophin, but also interrupted that which would otherwise result in persistence of active follicles and of estrogen secretion.

Pfeiffer (1936–1941) reported that in rat hypophyses, the steadily secreting male type is determined by early action of androgen during infancy. It now seems probable that this sex difference actually resides in the hypothalamus. The acyclic *constant-estrous* state which Pfeiffer obtained in female rats by prepubertal testis grafts was irreversible. Luteinization could not be induced by estrogen injection (1936) nor could cycles be established by progesterone therapy (1941). The acyclic *spontaneous persistent-estrous* state in intact females on the other hand, is reversible, as indicated above. Especially interesting in view of Pfeiffer's work, is the report that normal cycles and luteinization can be induced in spontaneously persistent-estrous rats (DA strain) by daily injection of small amounts of testosterone propionate (Marvin, 1948). It appears that in rats during infancy the action of androgen conditions differentiation of the hypothalamic "center" as an intrinsically acyclic mechanism. However, in an intact genetic female or in a male castrated in infancy and implanted with ovaries, the "center" differentiates as an intrinsically cyclic mechanism. Steady states may be induced later by certain environmental or internal factors, yet the cyclic potentiality tends to remain.

Whether only one or several neural centers are concerned in activation of the adenohypophysis cannot be judged from available evidence. Nor would we make the doubtful claim that all functions of the hypophysis depend on neural stimulation, even all gonadotrophic functions. There seems no question, however, that a revision of current views of gonadotrophin secretion is distinctly called for.

## SUMMARY AND CONCLUSIONS

In the preceding article it was shown that Dibenamine or atropine will block the mechanism which leads to release of LH from the adenohypophysis after estrogen injection. Since these drugs do not interfere with the glandular discharge of the hormone nor with its action on the ovary, the conclusion was reached that estrogen effects LH release at least partially by way of the nervous system, presumably the hypothalamus.

The existence in the cyclic rat of a similar mechanism and the chronologic relationship of this to the cycle are demonstrated in the present report, which concerns the results of injections of Dibenamine or of atropine at various hours of the day of proestrus.

Excepting preliminary experiments, the animals used were 68 4-day cyclic rats belonging to a strain in which ovulation normally occurs between 1 and 2:30 A.M. in the early morning after proestrus. When Dibenamine was injected during proestrus at 2 P.M. or earlier, ovulation was prevented or significantly retarded in most cases. Injection of atropine at these hours uniformly prevented preovulatory swelling or ovulation. Associated with these blocking effects of either drug was the failure both of the ovarian interstitial tissue to undergo the usual cholesterol depletion and of the corpora lutea of the preceding cycle to store cholesterol. The uteri usually remained distended the day after injection. In most cases the vaginal smear sequence was retarded significantly.

When either drug was injected at 4 P.M. during proestrus, ovulation proceeded without detectable interference apart from retardation in a few rats treated with Dibenamine. In the majority of cases the only suggestion of an effect upon LH release was an apparent delay of luteal cholesterol storage.

It is concluded that in this strain of rats, under our colony conditions, neurohumoral stimulation of the adenohypophysis occurs during proestrus between 2 and 4 P.M., 10 to 12 hours before ovulation. The demonstration that this chronologically limited neurohumoral stimulus is essential to adequate release of LH, clearly offers a means by which various features of the polyestrous cycle may be synchronized with environmental rhythms.

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# VARIABLES AFFECTING THE ASSAY OF TESTOSTERONE PROPIONATE USING THE SEMINAL VESICLE RESPONSE OF THE JUVENILE CASTRATED MALE RAT

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## INTRODUCTION

THE USE of the accessory sex organs as the criterion of response for the biological assay of androgens has been described by Korenchevsky 1932, Korenchevsky et al. 1932, Callow and Deansley 1935, Bulbring and Burn 1935, Deansley 1938, Greene and Burrill 1940, 1941, Hays and Mathieson 1945, and Mathieson and Hays 1945.

The procedure as outlined by the latter authors has been found a practical procedure for the assay of commercial preparations of testosterone propionate. However, the error of the method in the experience of this laboratory appeared relatively large and a study of some of the variables affecting the response was deemed advisable in order to determine whether any improvement in the precision of the assay could be obtained consistent with economy of materials, time and effort.

## METHODS

Immature rats of Wistar strain bred and raised in this laboratory were used. They were castrated at 28 to 29 days of age and within the weight range of 40 to 60 grams. The following technique was used for the removal and weighing of the seminal vesicles. After killing the rats with chloroform and exposing the internal sex organs, the seminal vesicles were stripped of their fibrous capsules and removed with fine forceps directly under a binocular dissecting microscope. The seminal vesicles were then washed in saline, excess fluid taken up by blotting briefly with filter paper and weighed on a Roller Smith balance sensitive to 0.2 mg. In the case of the assays shown in table 1, assays 7 to 12, the technique was as outlined by Mathieson and Hays (1945). Mazola oil (a brand of corn oil) was used as a diluent for the doses. Crystalline testosterone propionate U.S.P. XIII was used as a standard of reference, and the tests were carried out on market samples of testosterone propionate or on known dilutions of the reference standard. In the case of assays two weeks were allowed after castration before using the rats and three dosage levels of standard and sample were employed, the rats being distributed for dosing on the basis of equal body weight at the time

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of castration. The doses were injected intramuscularly with a 1 cc. tuberculin syringe carrying a 26 gauge $\frac{5}{8}$ inch needle, and subcutaneously with a 24 gauge $1\frac{1}{4}$ inch needle. Precautions were taken in the injections to prevent leakage of the doses by inserting the needles full length. The results were subjected to analysis of variance as outlined by Bliss and Marks (1939), the

TABLE 1. THE EFFECT OF THE VOLUME OF THE INJECTION AND METHOD OF ADMINISTRATION ON THE SLOPE OF THE REGRESSION LINE, VARIANCE AND CONFIDENCE LIMITS OF THE ASSAY

| Assay | No. of Rats | Slope b | Variance $s^2$ | Confidence Limits in Per Cent Sm. ×1.96 |
|---|---|---|---|---|
| | 0.1 cc. Subcutaneously | | | |
| 1 | 42 | 13.95 | 17.86 | 65.4 to 153.0 |
| 2 | 48 | 7.97 | 18.12 | 49.7 to 201.2 |
| 3 | 48 | 8.30 | 18.50 | 49.9 to 200.3 |
| 4 | 48 | 8.06 | 20.20 | 43.9 to 227.7 |
| 5 | 48 | 9.05 | 16.60 | 53.9 to 185.7 |
| 6 | 48 | 9.37 | 20.24 | 51.4 to 194.2 |
| Mean | | 9.36 | 18.61 | |
| | 0.2 cc. Subcutaneously | | | |
| 7 | 48 | 9.25 | 13.97 | 59.0 to 169.0 |
| 8 | 48 | 9.30 | 7.74 | 67.7 to 147.8 |
| 9 | 48 | 14.05 | 13.13 | 71.4 to 140.0 |
| 10 | 48 | 8.25 | 8.62 | 60.7 to 164.2 |
| 11 | 48 | 6.93 | 9.28 | 55.0 to 181.9 |
| 12 | 48 | 7.04 | 8.61 | 58.0 to 172.4 |
| Mean | | 9.14 | 10.23 | |
| | 0.1 cc. ×2 Intramuscularly | | | |
| 13 | 48 | 12.09 | 3.93 | 80.7 to 123.9 |
| 14 | 48 | 11.54 | 3.04 | 81.9 to 122.0 |
| 15 | 48 | 12.45 | 1.60 | 87.6 to 114.2 |
| 16 | 48 | 12.62 | 3.12 | 83.3 to 120.0 |
| 17 | 48 | 10.38 | 3.01 | 80.3 to 124.6 |
| 18 | 48 | 12.96 | 5.08 | 79.7 to 125.4 |
| Mean | | 12.01 | 3.30 | |

values for the slope of the regression line and the variance of the assay being used as indicators of the estimate of the precision of the assay.

## RESULTS

Table 1 shows the results obtained in the assay of market samples of testosterone propionate employing first the injection technique as outlined by Mathieson and Hays (1945) of using 0.1 cc. subcutaneously, secondly, of using 0.2 cc. subcutaneously and finally of using intramuscular injections and giving the dose in a volume of 0.1 cc. into the muscle of each hind leg (0.1 cc.×2 intramuscularly). It is seen that increasing the volume of the injection from 0.1 cc. to 0.2 cc. subcutaneously tends to decrease the variance of the assay (column 4) and the precision as reflected in the calculated confidence limits (column 5). When the injections are given intramuscularly the slope of the regression line is within the same range as for subcutaneous

injection, but the variance of the assay is markedly decreased and the estimate of the confidence limits reduced to values which are considered reasonable for most biological assays. Thus it is seen that intramuscular administration of the doses improves the precision of the assay of testosterone propionate.

In order to obtain a direct comparison of the two methods of administration the tests shown in table 2 were performed using known dilutions of the testosterone propionate reference standard. In the first group of assays (columns 2 and 3) the results obtained using 24 rats (8 per dosage level) and 0.1 cc. subcutaneously were compared with the results obtained using 24 rats (8 per dosage level) and 0.1 cc.×2 intramuscularly (columns 4 and 5). The dosage levels used for

TABLE 2. THE EFFECT OF SUBCUTANEOUS AND INTRAMUSCULAR ADMINISTRATION ON THE SLOPES, VARIANCES AND SENSITIVITIES

| Assay | Slope b | Variance $s^2$ | Slope b | Variance $s^2$ | Ratio in per cent Intramuscular / Subcutaneous | Confidence Limits of Ratio in per cent Sm.×1.96 |
|---|---|---|---|---|---|---|
| | 0.1 cc. Subcutaneously | | 0.1 cc.×2 Intramuscularly | | | |
| 1 | 15.44 | 12.21 | 10.96 | 3.06 | 36.7 | 28.3 to 49.0 |
| 2 | 13.95 | 24.51 | 12.79 | 4.58 | 19.3 | 13.2 to 28.2 |
| 3 | 15.94 | 17.12 | 13.45 | 5.00 | 28.3 | 21.0 to 38.1 |
| 4 | 15.44 | 18.11 | 13.28 | 2.09 | 33.9 | 25.2 to 45.6 |
| Mean | 15.20 | 18.00 | 12.62 | 3.63 | 30.1 | 25.7 to 35.2 |
| | 0.1 cc.×2 Subcutaneously | | 0.1 cc.×2 Intramuscularly | | | |
| 1 | 13.12 | 8.66 | 11.79 | 1.84 | 55.4 | 41.3 to 74.3 |
| 2 | 10.79 | 7.62 | 10.29 | 4.12 | 62.5 | 42.5 to 91.9 |
| 3 | 9.13 | 6.40 | 12.62 | 1.31 | 54.7 | 41.1 to 72.8 |
| 4 | 15.45 | 8.29 | 11.46 | 2.26 | 47.1 | 36.5 to 60.8 |
| Mean | 12.12 | 7.75 | 11.54 | 3.38 | 53.2 | 45.8 to 61.7 |

the subcutaneous injections were 0.2 mgm., 0.4 mgm. and 0.8 mgm. respectively, while the dosage levels for the intramuscular injections were 0.05 mgm., 0.10 mgm. and 0.20 mgm. respectively. It is seen that the slopes of the regression lines for the two routes of administration are within the same range, but the variance of the test is definitely higher (mean 18.00) in the case of the subcutaneous injections than in the case of the intramuscular administration (mean 3.63). In column 6, the ratios of the activity of the intramuscular injections to the subcutaneous injections are shown. It is seen that the rats are considerably less sensitive to the subcutaneous injection (mean 30 per cent) or it requires approximately three times as much testosterone propionate subcutaneously to produce a comparable response intramuscularly. The estimate of the confidence limits of the ratio is shown in the last column.

In order to determine if the division of the doses subcutaneously would produce results comparable to the intramuscular administration the tests shown in the second group of assays in table 2 were performed. The same number of rats per assay and the same dosage

levels of testosterone propionate were used as in the first group. In the case of the subcutaneous administration the injections were made in amounts of 0.1 cc. into the loose subcutaneous tissue on opposite sides of the rat. In this group of assays the variances of the tests (column 3) are lower (mean 7.75) using 0.1 cc.×2 subcutaneously than the variance (mean 18.00) using 0.1 cc. subcutaneously in the first group. The ratios of the activity (mean 53.2%) shown in column 6 of intramuscular to subcutaneous administration indicate that the division of the doses subcutaneously increases the sensitivity, however, it required approximately twice as much testosterone propionate at 0.1 cc.×2 subcutaneously as it did at 0.1 cc.×2 intramuscularly to produce comparable responses.

TABLE 3. THE EFFECT OF THE METHOD OF ADMINISTRATION ON THE PRECISION OF THE ASSAY

| Method of Administration | No. of Assays | $(s/b)^2$ | No. of rats for Comparable precision |
|---|---|---|---|
| 0.1 cc.×2 Intramuscularly | 6 | .023 | 1.0 |
| 0.1 cc.×2 Subcutaneously | 4 | .051 | 2.2 |
| 0.2 cc. Subcutaneously | 6 | .123 | 5.3 |
| 0.1 cc. Subcutaneously | 6 | .212 | 9.3 |

Table 3 is a summary of the results obtained with the different methods of injection employed using the ratio of the value of the slope to the variance as an indicator of the estimate of precision. Since the method using 0.1 cc.×2 intramuscularly gave the lowest value for $(s/b)^2$ (column 3) this was used as a basis of comparison and was given the value 1.0 (column 4), while the remaining methods of injection are expressed in terms of this indicating the number of rats required in an assay for comparable precision. These results show that in the intramuscular administration of the doses there is considerable economy of rats and the division of the doses subcutaneously is quite superior to single injections with respect to the number of rats required for comparable precision.

The influence of the time of autopsy on the seminal vesicle response to testosterone propionate administered intramuscularly (0.1 cc.×2) is shown in figure 1. Each point on the graph represents the mean seminal vesicle weight of 10 rats killed 24, 48, 72 and 96 hours after injection at the dosage level of 0.05 mgm., 0.10 mgm. and 0.20 mgm. respectively. It is seen that the maximum response is obtained at 72 hours after injection on the two lower dosage levels, while at the higher dosage level the response is maintained until 96 hours after injection. These results are similar to those reported by Hays and Mathieson (1945) indicating that the time relationship of the response for the intramuscular injection of the doses are the same as those for the subcutaneous injection of the doses.

Hooker (1942) has shown using male rats castrated at birth that the sensitivity of such rats to testosterone increased markedly at 40, 50 and 60 days of age, this being the age when the accessory sex glands normally undergo puberal changes. Since it is difficult to obtain a sufficient number of immature rats for castration at one time to perform an assay, it is customary to assemble rats at various post castration ages as recommended by Hays and Mathieson (1945). The effect of testosterone propionate on the seminal vesicle response of the juvenile castrated rats over the above age range was not well covered by the above workers and it was considered advisable to de-

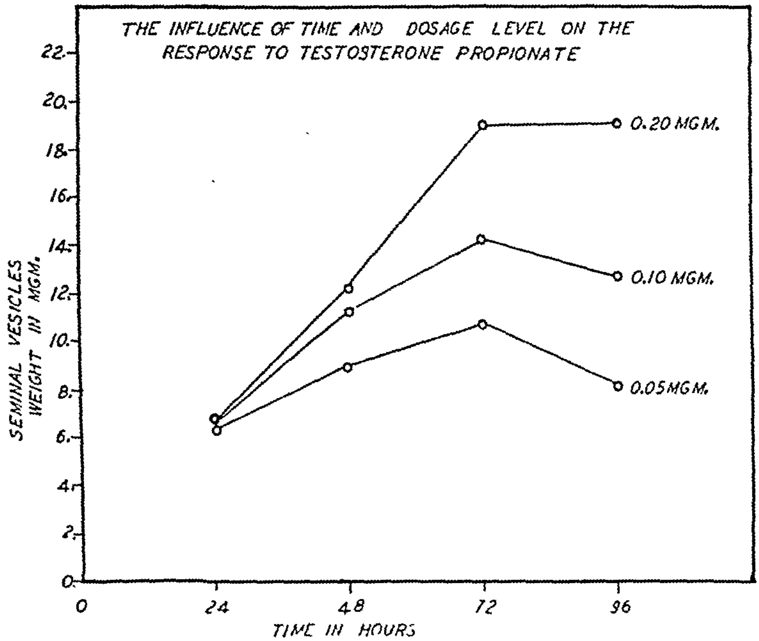


FIG. 1. The influence of the time of autopsy and dosage level on the response of testosterone propionate administered intramuscularly.

termine if the alterations in sensitivity reported by Hooker (1942) might account for some of the variations in response encountered in assembling rats for assay of the various post castration ages.

The following experiment was designed to determine this point. Fifteen rats were castrated and 10 of these were injected immediately with 0.2 mg. of testosterone propionate (0.1 cc. $\times$2 intramuscularly) and 5 were kept as controls. Seventy-two hours later the group was killed and the seminal vesicle removed and weighed. In the same manner other groups of 15 rats were castrated, except before injection 7, 14, 21, etc. days elapsed after castration. In this way the seminal vesicle response to 0.2 mgm. of testosterone propionate and untreated controls were followed over the age range from 28 to 98 days. The results are shown in figure 2. It is seen that the rats tend to be more

sensitive to testosterone propionate during the first two weeks after castration, but after this time the response is relatively uniform. Applying the $X^2$ tests to the mean of all the points it is seen that they do not deviate significantly from a straight line $X^2=0.98$. At 10 degrees of freedom $X^2=18.03$ p at 0.05. However, when the first two points are eliminated the fit to a straight line is considerably improved and does not differ from the results obtained for the untreated con-

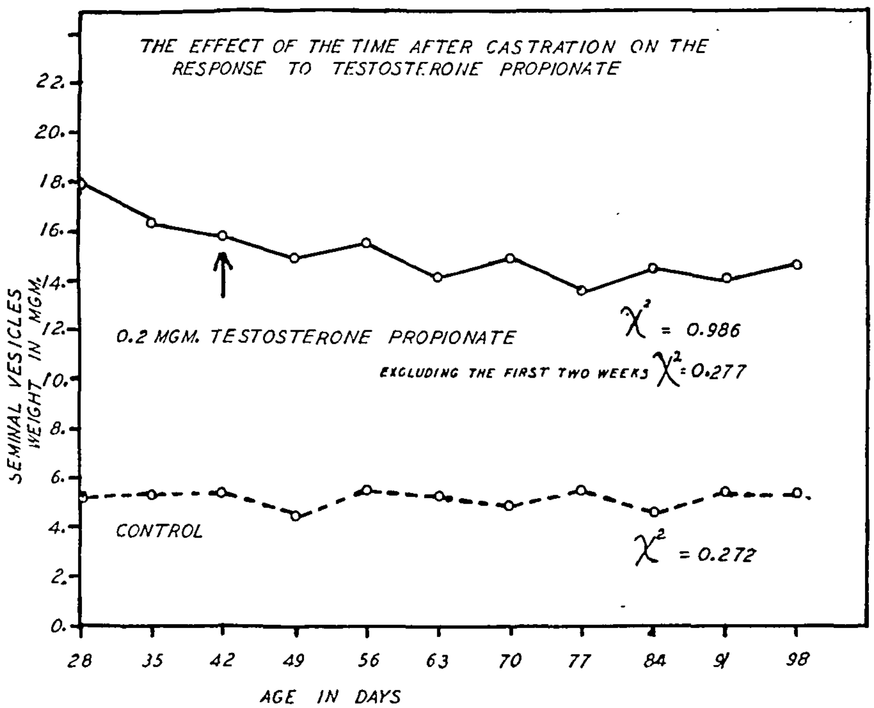


FIG. 2. The effect of the time after castration on the response to 0.2 mgm. of testosterone propionate administered intramuscularly—solid line and on untreated controls—broken line.

trols. No definite break or trend is indicated after the first two weeks post castration. These results indicate that, except for the first two weeks after castration, the sensitivity of rats castrated at 28 days of age to testosterone propionate does not change significantly and the use of rats for assay of various post castration ages does not affect the variations encountered in the response.

## DISCUSSION

The assay of testosterone propionate using the seminal vesicle response of the juvenile castrated male rat has been found to be a practical procedure for checking the potency of commercial samples of this androgen. The advantage of using intramuscular injection of the doses is shown in tables 1, 2 and 3. A better utilization of the testosterone propionate is indicated from the lower value of the variance and the

greater sensitivity in the intramuscular injections than in the subcutaneous injections, table 2. Leathem (1948) has shown recently that the response to testosterone propionate injected subcutaneously was not influenced by the volume of oil and that the subcutaneous and intramuscular routes of administration increased accessory sexual organ weight equally well. The difference between these results and those reported here may be due to the use by Leathem (1948) of uncastrated immature rats and including the weight of the coagulating glands with the weight of the seminal vesicles. Diluents other than Mazola oil for the dosage levels were not tried and this may be a factor in the response as shown by Crafts (1942) in the seminal vesicle response of mice to sesame oil and by Albrieux and Prego (1943) on the activity of estrone in various oils as diluents.

The results obtained in the assays shown in table 1 were subjected to factorial analysis in order to determine if linear dosage response relationships existed over the dosage range used. The variance ratio value for the slope of the dosage response line was highly significant in all the assays and in none of the assays was there any indication from the variance ratio value of lack of parallelism between the slope of the regression line of the standard and unknown. The variance ratio value also showed no significant degree of curvature or opposed curvature in the regression lines of any of the assays.

The time relationships of the response (figure 1) to intramuscular administration are similar to those reported by Hays and Mathieson (1945) for subcutaneous administration. The maximum response is obtained at approximately 72 hours after injection and there is a better differentiation of the response at this time than at 48 hours after injection as recommended by Greene and Burrill (1941). The assembling of rats of various castration ages is justified in accordance with the results shown in figure 2. It is evident that the sensitivity of the rats castrated at 28 days of age does not change significantly as reported by Hooker (1942) for rats castrated at birth.

This method of assay was not found applicable for checking the potency of commercial samples of methyl testosterone or testosterone on account of relatively large amounts required to produce a response (5 to 10 mgm.) and the low solubility of these products in oil presented difficulties in obtaining convenient volumes for injection (unpublished results.).

## SUMMARY

In a study of the variables affecting the assay of testosterone propionate using the seminal vesicle response of the juvenile castrated rat it was found that the precision and sensitivity of the assay could be improved some by the division of the doses administered subcutaneously and considerably by intramuscular administration of the doses. Except for the first two weeks after castration, the sensi-

tivity of rats castrated at 28 days of age to testosterone propionate does not change significantly and the variations in response of the seminal vesicles are not influenced by assembling rats of various castration ages.

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# THE ADMINISTRATION OF ADRENOCORTICOTROPHIC HORMONE TO NORMAL HUMAN SUBJECTS. THE EFFECT OF THE LEUCOCYTES IN THE BLOOD AND ON CIRCULATING ANTIBODY LEVELS

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THE FIRST suggestion that the adrenals might be concerned in the general defence reaction of the body followed observations that these organs sometimes showed marked pathological changes during acute infective and toxic episodes. Several studies were made in which the effect of adrenalectomy on antibody titres was investigated. Reports were contradictory. Thus Hektoen and Curtis (1915) found that the level of circulating antibodies in dogs was not altered by bilateral operation and Gates (1918) reported that the rate of antibody formation by rabbits was unaffected by previous partial adrenalectomy. Jaffe and Marine (1924), on the other hand, obtained higher titres after bilateral adrenalectomy when the animals had been immunized three weeks after operation, but noted that at six weeks there was no difference in the response between operated and unoperated rabbits. Their results were in direct contradiction to those of Perla and Marmorston-Cottesman (1928) who noted decreased rates of antibody production in rats injected during the five weeks following operation, this decrease being most marked in the first week.

As the chemistry and physiology of the adrenal cortex were made clearer it became possible to work on animals under more nearly physiological conditions. Fox and Whitehead (1936) were the first to try the effect of injecting adrenal cortical extract into experimental animals. They reported an increased rate of production of antibodies following injection of the hormone but found that control animals subsequently attained much the same titres.

These experiments were repeated and extended by Chase, White and Dougherty (1946). Using cortical extracts, synthetic cortical hormones and adrenocorticotrophin (ACTH), they have worked both with normal animals and with adrenalectomised animals maintained on desoxycorticosterone acetate (DCA). They have produced evi-

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dence, not only that the cortical hormones increase antibody levels when given during the immunisation period, but also that they can effect an immediate release of stored antibody into the circulation. Recently an attempt was made by Eisen *et al.* (1947) to measure the first of these effects more precisely by using an accurate chemical method of antibody determination. They were unable to show that the production of antibody was affected by the injection of cortical hormones or that it was dependant on the presence of the adrenal. They did, however, partially confirm the findings of Chase, White and Dougherty in that they sometimes noted transient increases of antibody nitrogen immediately after injection of cortical extracts.

The experiments described below were designed, to elicit this latter effect in human subjects following stimulation of the adrenal cortex by ACTH. Staphylococcal toxoid was chosen for the immunising agent, as the estimation of anti-haemolysin is quick and can be carried to a finer end-point than titrations involving agglutinations. The differential leucocyte counts which are also reported were a partial check on the activity of the hormone preparation, one of the results of adrenal cortex stimulation being a decrease in the numbers of circulating lymphocytes (Dougherty and White, 1944) and eosinophils (Forsham *et al.* 1948).

## TECHNICAL METHODS

*Antibody to staphylococcus alpha-haemolysin.* The potency of the toxin was measured initially against a sample of international standard anti-serum and thereafter occasionally checked against a sub-standard serum. In titrating the unknown sera the toxin was diluted with 0.85% NaCl so that 1 ml. contained either 1 L.H. unit or $\frac{1}{5}$ L.H. unit. The latter dilution was used when the sera were of low potency and of insufficient quantity to allow accurate titration at the higher level. In any one experiment all the sera were titrated simultaneously, using the same batch of diluted toxin. After heating for 40 minutes at 56°C. to inactivate complement, the sera were incubated with a known volume of a rabbit red cell suspension to absorb the red cell agglutinins and final dilutions were then made with saline from the supernatant fluid. 1 ml. of each dilution was incubated with 1 ml. of toxin for 1 hour at 37°C. and 0.5 ml. of a 5% suspension of washed rabbit cells added to each tube. After a further 60 minutes incubation the tubes were kept at about 4°C. overnight. They were then centrifuged and the end-point taken as mid-way between the dilution of serum showing no haemolysis and that showing trace or partial haemolysis.

*Agglutinins to the typhoid "H" antigen.* The antigen was a formalized suspension of E. typhosa "Buzzard" containing approximately 400 million organisms per ml. Doubling dilutions of sera in saline were made and 1 ml. of each dilution, together with 1 ml. of antigen suspension, was incubated at 56°C. After 6 hours the end-point was taken as the highest serum dilution showing complete agglutination.

*Total leucocyte counts and eosinophil counts* were made according to the method described by Randolph (1944), using a water-propylene glycol mixture as the diluting fluid. For the total leucocytes, the cells contained in the

four large corner squares in each of four or eight chambers were counted. For the eosinophils the cells within the entire ruled area of at least 10 chambers were counted.

Smears for the *differential cell counts* were made on coverslips and stained by Wright's method. Areas extending outwards from the tail of the smear were chosen, having regard to even distribution of the leucocytes, lack of fragmentation and thinness of the film. The number of cells counted from each sample is given in the tables of results.

*Haematocrits* were measured in Wintrobe tubes after spinning for 45 minutes at *ca.* 3000 r.p.m. Two tubes were filled and the average of the two readings is reported. The speed of centrifugation was checked occasionally by reading the haematocrits after 30, 45 and 60 minutes.

*Plasma and serum protein values* were determined by the method of Jacobsen and Lindstrøm-Lang (1940), using the Philips, van Slyke *et al.* (1945) nomogram for the conversion of R.D. to gms. protein/100 ml. The standards used ($CuSO_4$ solutions) were also taken from the latter communication and were checked by direct weighing in a 25 ml. capacity specific gravity bottle. The density of whole blood was measured in some experiments and packed cell volumes calculated from this usually agreed with those determined by direct measurement.

*Glycogenic corticoids and 11-oxy steroids* in the urine were measured by Dr. E. H. Venning and are reported in a separate communication (1948). Urinary corticoids were determined by bioassay according to the method of Venning *et al.* (1946) and 11-oxy steroids by the method of Talbot *et al.* (1945).

### SUBJECTS

The subjects of the experiments were healthy normal adults who had been inoculated with commercial preparations of typhoid vaccine and staphylococcal toxoid.

Subject "H"—female, aged 29 years—received combined T.A.B. vaccine and tetanus toxoid in 1941 and a routine course of staphylococcal toxoid (0.05 ml. increasing to 1.0 ml.) during March 1947. This was followed by a single injection of 2.0 ml. staphylococcal toxoid on June 16th. The highest values for anti-haemolysin were found in April 1947 and were between 11 and 12 Provisional International Units (P.I.U.) per ml. serum.

Subject "Rs"—male, aged 39 years—received combined T.A.B. vaccine and tetanus toxoid in 1944 followed by several "booster" injections, the last being given in July 1947. He received the same routine course of staphylococcal toxoid as subject H, in May 1947. The highest recorded values for anti-haemolysin were found in June 1947 to be about 10 P.I.U./ml.

Subject "Ha"—male, aged 32 years—received combined T.A.B. vaccine and tetanus toxoid prior to 1945 with the last injection in the winter of that year, and a course of staphylococcal toxoid during September and the beginning of October 1947. After a preliminary injection of 0.25 ml. four injections of 0.5 ml. and one of 1.0 ml. were given at weekly intervals. Measurements of the anti-haemolysin values for the serum of this subject were not made until shortly before the start of the experiment.

Blood samples for control leucocyte counts were also taken from three other individuals. Subject "Gr" was a male, aged 31 years, and Subject "V," a female aged 29 years. Subject "Go" was an apparently healthy

airman aged 24 years. He was later found to have an impaired glucose tolerance but was otherwise normal.

## EXPERIMENTAL MATERIAL

Adrenocorticotrophin was supplied by the Armour Laboratories. Two preparations were used, both of which had been made from hog pituitary glands by a modification of the method of Sayers *et al.* (1943). Batch 32D, which was used in experiments 1 to 4, contained 0.1 unit pitressin/mg. but Batch 37KE, which was used in experiment 5, contained only a trace of this material. Other pituitary hormones were absent or present only in negligible quantities. Both batches were assayed by the Armour Laboratories against their standard preparation LA-1-A. Batch 32D was found to have 70%, and Batch 37KE 50%, of the adrenocorticotrophic activity of this preparation.

The material supplied was a powder and was dissolved in saline prior to intramuscular injection. Batch 37KE had previously been sterilised and Batch 32D was sterilised by boiling for 15 minutes immediately after the solution had been made. The dissolved material was occasionally stored overnight before using and was then kept frozen at −20°C.

## RESULTS

Adrenocorticotrophin was given to normal subjects on five occasions. In addition to these experiments various control leucocyte counts were made at different times of day. As the preparation of

TABLE 1. THE DIURNAL VARIATION IN LEUCOCYTE COUNTS UNDER NORMAL CONDITIONS AND AFTER THE INJECTION OF PITRESSIN

| Subject & Date | Time | Leucocyte Counts Per Cubic Millimetre | | | | | Number of Cells Examined |
|---|---|---|---|---|---|---|---|
| | | Total | Neutrophil | Lymphocyte | Eosinophil (Direct) | Monocyte | |
| "V" 17/ 9/47 | 10:00 A.M. | 5,910 | 3,460 | 1,790 | 188 | 420 | 2,000 |
| | 2:30 P.M. | 6,540 | 3,870 | 2,100 | 195 | 510 | 2,000 |
| | 5:00 P.M. | 7,320 | 4,300 | 2,420 | 245 | 600 | 2,000 |
| | 8:00 P.M. | 8,490 | 5,600 | 2,380 | 238 | 476 | 2,000 |
| "H" 22/ 9/47 | 10:00 A.M. | 8,930 | 5,370 | 2,640 | 164 | 652 | 2,000 |
| | 1:30 P.M. | 9,550 | 6,280 | 2,460 | 153 | 572 | 2,000 |
| | 4:45 P.M. | 11,200 | 6,820 | 3,270 | 150 | 851 | 2,000 |
| | 8:45 P.M. | 13,540 | 8,440 | 3,840 | 250 | 866 | 2,000 |
| "Gr" 15/10/47 | 10:00 A.M. | 5,560 | 2,420 | 2,650 | 89 | 378 | 1,000 |
| | 4:30 P.M. | 6,410 | 3,250 | 2,550 | 123 | 500 | 1,000 |
| | 8:00 P.M. | 6,905 | 3,220 | 3,040 | 144 | 456 | 1,000 |
| "H" 8/11/47 | 10:00 A.M. | 10,010 | 5,200 | 3,750 | Not done | 900 | 2,000 |
| | 12:15 P.M. | 9,900 | 6,170 | 2,690 | Not done | 841 | 2,000 |
| | 2:45 P.M. | 10,120 | 6,320 | 3,028 | Not done | 668 | 2,000 |
| "Go" 14/ 4/48 | 10:00 A.M. | 5,530 | 3,750 | 1,140 | 122 | 514 | 2,000 |
| | 12 noon | 6,280 | 3,750 | 1,800 | 137 | 559 | 2,000 |
| | 4:00 P.M. | 6,960 | 4,130 | 1,930 | 188 | 689 | 2,000 |
| | 8:00 P.M. | 7,630 | 5,370 | 1,610 | 178 | 466 | 2,000 |
| 20 units pitressin, intramuscularly—5 units at 10:05 A.M., 12 noon, 2:00 P.M., 3:00 P.M. | | | | | | | |
| "H" 10/ 9/47 | 10:00 A.M. | 9,420 | 6,360 | 2,050 | 109 | 791 | 1,000 |
| | 5:00 P.M. | 13,620 | 9,940 | 2,550 | 75 | 926 | 1,000 |
| 20 units pitressin, intramuscularly—10 units at 10:05 A.M., 11:00 A.M. | | | | | | | |
| "H" 15/ 9/47 | 10:00 A.M. | 10,480 | 7,200 | 2,350 | 141 | 776 | 1,000 |
| | 12:30 P.M. | 11,300 | 8,500 | 1,840 | 53 | 870 | 1,000 |
| | 4:00 P.M. | 12,970 | 9,090 | 2,780 | 94 | 986 | 1,000 |

hormone initially available (32D) contained appreciable amounts of posterior pituitary contaminant, some counts were also made on one subject following the injection of 20 units of pitressin. 200 mgs. of the corticotrophin Batch 32D assayed as containing this amount of pitressin.

All these control findings are summarised in Table 1.

In general the findings confirmed those of Elmadjian and Pincus (1946) with regard to the lymphocytes and those of Rud (1947) with regard to the eosinophils. The latter author has drawn attention to the marked variation which may occur even from minute to minute in the absolute eosinophil count. The range of normal levels noted by

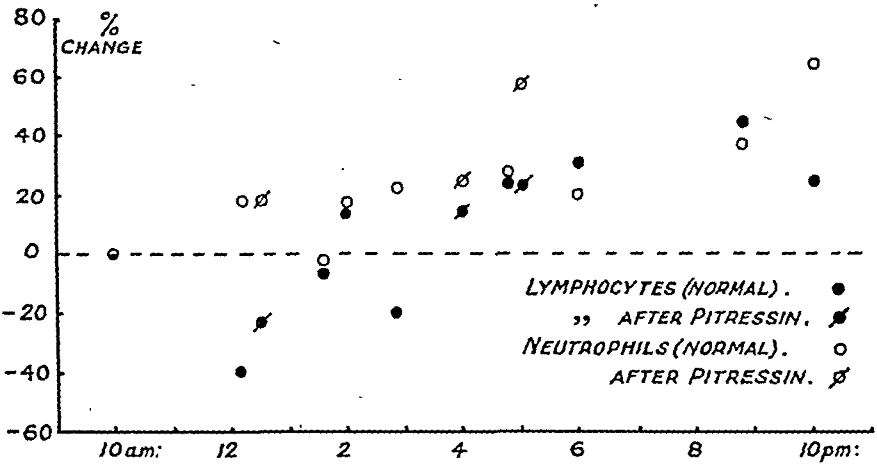


FIG. 1. Diurnal variations in lymphocyte and neutrophil counts in a female subject (H) on five separate control days. (See also Tables 1 and 6).

Percentage changes plotted in this and subsequent figures have been calculated from values obtained at or about 10 A.M.

him was quite similar to that shown in Table 1, although the variations in his subjects were rather more marked.

This variability of the eosinophil count makes it difficult to decide whether the fall noted after giving pitressin to subject "H" should be attributed to the effect of the hormone or not. If on these two occasions stimulation of the adrenal occurred as part of the reaction to pitressin it was not of the same degree as that induced on other occasions by injection of corticotrophin.

In Figure 1 the neutrophil and lymphocyte changes for five control days in Subject "H" are charted.

The early fall and later steady rise in the lymphocytes reported by Elamadjian and Pincus is well shown here. With regard to the monocytes, it can only be stated that the variations are of the same degree as those noted later in the corticotrophin experiments. It is unlikely that sufficient cells were examined to establish the significance of these variations.

TABLE 2. *Experiment 1.* EFFECT OF 40 MG. ACTH ON THE BLOOD PICTURE OF A FEMALE SUBJECT (H)

| Date | Time | Leucocyte Counts Per Cubic Millimetre | | | | | Number of cells examined | Haemato-crit Vol. % | Serum proteins gms./100 ml. | Antihae-molysin PIU/ml.[1] serum | Agglutinin Titer |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Neutro-phil | Lympho-cyte | Eosino-phil (Direct) | Mono-cyte | | | | | |
| 8/7/47 | 10:20 A.M.[2] | 8,250 | 4,570 | 2,900 | 115 | 611 | 1,000 | 46.4 | 7.3 | 6.0 | 1/40 |
| 8/7/47 | 11:25 A.M. | 7,460 | 4,400 | 2,570 | 75 | 527 | 1,000 | 43.9 | 7.0 | 5.7 | 1/40 |
| 8/7/47 | 12:40 P.M. | 12,240 | 8,940 | 2,780 | 26 | 404 | 1,500 | 44.7 | 7.3 | 6.0 | 1/40 |
| 8/7/47 | 2:25 P.M. | 11,720 | 9,550 | 1,680 | 12 | 390 | 1,500 | 44.7 | 7.2 | 5.8 | 1/20 |
| 8/7/47 | 5:00 P.M. | 12,870 | 9,570 | 2,670 | 17 | 579 | 1,000 | 45.3 | 7.5 | 7.0 | 1/20 |
| 8/7/47 | 8:30 P.M. | 13,120 | 8,470 | 3,700 | 129 | 700 | 1,000 | 45.2 | 7.2 | 5.4 | not done |
| 9/7/47 | 11:20 A.M. | 9,390 | 6,350 | 2,360 | 81 | 730 | 1,000 | 43.4 | 7.0 | 5.1 | 1/40 |

[1] PIU indicates "provisional international unit."
[2] ACTH (32D) ≡28 mg. Armour standard LA-1-A given immediately after withdrawl of this sample.

TABLE 3. *Experiment 2.* EFFECT OF 100 MG. ACTH ON THE BLOOD PICTURE OF A MALE SUBJECT (Rs)

| Date | Time | Leucocyte Counts Per Cubic Millimetre | | | | | Number of cells examined | Haemo-crit Vol. % | Plasma proteins gms./100 ml. | Antihae-molysin PIU/ml. serum | Agglutinin Titre |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Neutro-phil | Lympho-cyte | Eosino-phil (Direct) | Mono-cyte | | | | | |
| 1/9/47 | 1:00 P.M. | 4,780 | 2,460 | 1,800 | 102 | 344 | 2,000 | not done | not done | 5.7 | 1/80 |
| 2/9/47 | 10:00 P.M.[1] | 6,160 | 3,030 | 2,290 | 149 | 570 | 1,000 | 41.8 | 7.4 | 6.2 | 1/80 |
| 2/9/47 | 12 noon | 5,410 | 3,840 | 1,090 | 57 | 303 | 1,000 | 39.7 | 7.0 | 5.7 | 1/80 |
| 2/9/47 | 1:50 P.M. | 10,220 | 9,110 | 730 | 18 | 290 | 1,000 | 39.3 | 7.1 | 6.0 | 1/160 |
| 2/9/47 | 4:00 P.M. | 8,730 | 7,730 | 770 | 6 | 183 | 1,000 | 39.2 | 7.3 | 6.2 | 1/80 |
| 2/9/47 | 6:00 P.M. | 9,410 | 7,740 | 1,280 | 16 | 352 | 1,000 | 40.1 | 7.4 | 5.5 | 1/80 |
| 2/9/47 | 8:10 P.M. | 9,800 | 7,370 | 2,050 | 44 | 314 | 1,000 | 41.2 | 7.4 | 5.2 | 1/80 |
| 3/9/47 | 6:00 P.M. | 7,000 | 4,020 | 2,070 | 148 | 640 | 1,000 | not done | not done | 5.7 | 1/80 |

[1] ACTH (32D) ≡70 mg. Armour standard LA-1-A 50 mg. given immediately after withdrawal of this sample, the remainder one hour later.

*Experiments with Adrenocorticotrophin*

*Experiments 1 to 3.* In these an attempt was made to induce a sudden and pronounced increase in adrenal cortical activity. The quantity of hormone that could be injected at one time was limited by the pitressin content of the preparation. To minimize the effect of this the larger doses of corticotrophin were divided and injected over periods of 1–2 hours. In spite of this, one subject ("Rs", experiment 2) showed a very marked reaction, presumably due to the posterior pituitary contaminant. After receiving two injections of 50 mgs. corticotrophin, separated by an interval of 60 minutes, his resting blood pressure rose in 30 minutes to 180/120 and his pulse rate, normally around 80, fell to 65. He had diarrhoea and marked abdominal cramps. One hour after the injection his blood pressure had fallen to 134/88 and an hour later he had apparently recovered. In this experiment, then, one cannot exclude the possibility that the reaction to pitressin in itself produced a sufficient "stressful" situation to stimulate the adrenal cortex. In experiment 1 where 40 mgs. of the same preparation were used and in experiment 3 with 200 mgs. no serious reaction occurred. It was noted, however, that the pallor which occurred following the injections was of much longer duration and more intense than that following the injection of similar or larger quantities of purified posterior pituitary hormone.

The results of these experiments are shown in Tables 2–4.

The findings in these three experiments were very similar and are illustrated by Figure 2, where the results from experiment 3 are charted.

In the white cell counts the eosinophils showed the most marked change. The fall seen in experiment 3 occurred in all experiments and is apparently related in both degree and duration to the quantity of hormone administered. While the number of lymphocytes also decreased in this and other experiments, this was not more pronounced with the larger doses of corticotrophin. These cells always returned more quickly to normal than the eosinophils. The rise in the neutrophil count is similar to that produced by the injection of other foreign proteins. Like the lymphocyte response, it is variable in degree and duration.

The serum protein and haematocrit values are of interest. Although the heamatocrit fell by 11%, indicating an increase in plasma volume, the serum protein values were not decreased. They were on the contrary slightly raised. In the first two experiments also, the haematocrits fell, but not more than is usual in the course of the day.

Values for anti-haemolysin are also shown in Figure 2. They are decreased slightly in parallel with the haematocrit, but the changes noted are of doubtful significance. In the first experiment with 40 mgs. corticotrophin there was a definite but slight increase 6½ hours after the injection (Table 2), whereas in the 100 mg. experiment

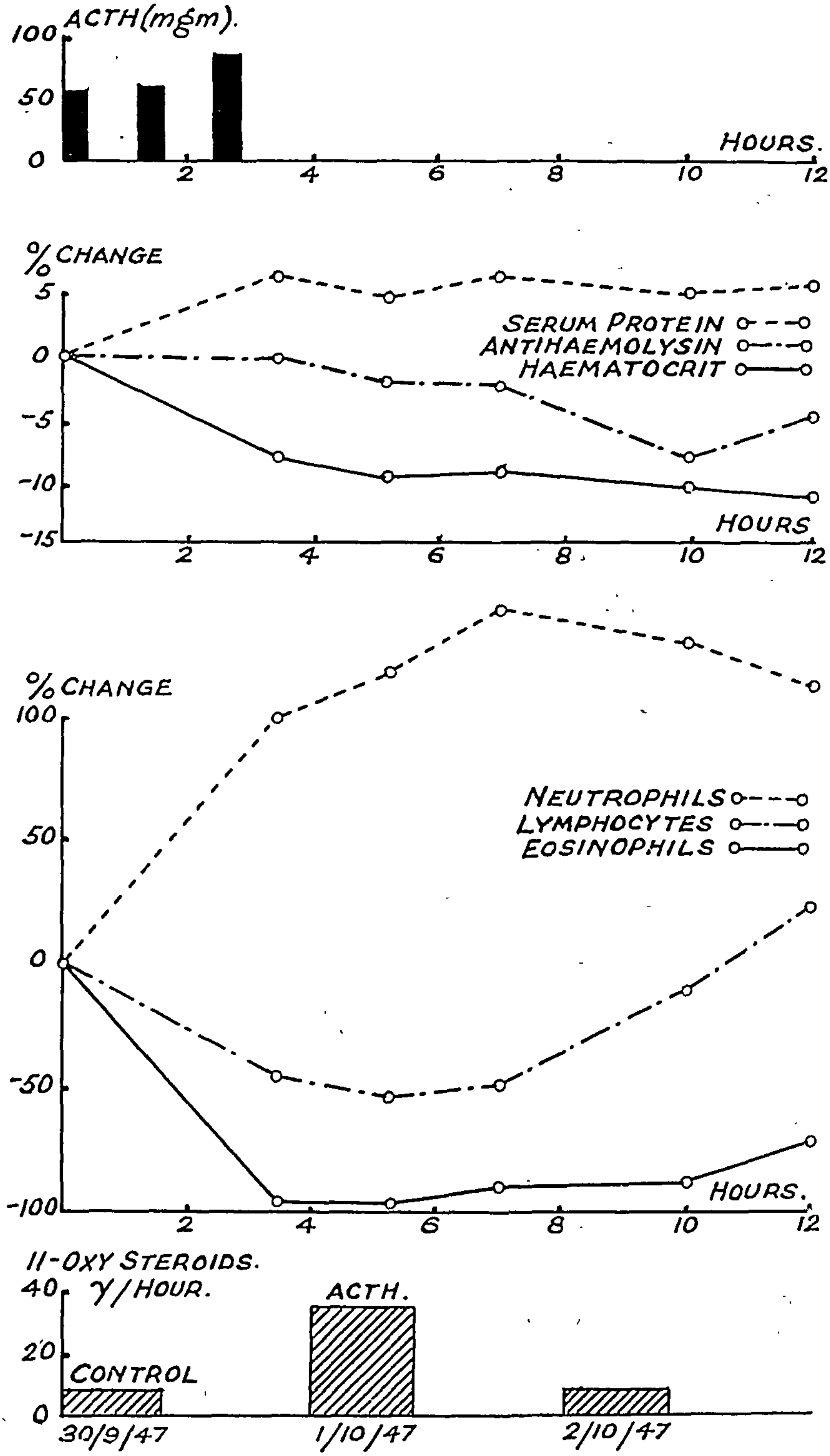


FIG. 2. Experiment 3. Suject H. The effect of 200 mg. ACTH (see Table 4).

(Table 3), a fall of about the same extent was noted at 10 hours. In these three experiments agglutinins to the typhoid "H" antigen were also titrated and showed no marked change. In experiment 2 (Table

TABLE 4. *Experiment 3.* EFFECT OF 200 MG. ACTH ON THE BLOOD PICTURE OF A FEMALE SUBJECT (H)

| Date | Time | Leucocyte Counts Per Cubic Millimetre | | | | | Number of cells examined | Haemocrit Vol. % | Serum proteins gms./100 ml. | Antihaemolysin PIU/ml. serum | Agglutinin Titre |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Neutrophil | Lymphocyte | Eosinophil (Direct) | Monocyte | | | | | |
| 1/10/47 | 9:30 A.M.[1] | 8,000 | 5,130 | 2,240 | 103 | 464 | 2,000 | 46.3 | 7.4 | 4.2 | 1/30 |
| 1/10/47 | 1:15 P.M. | 12,150 | 10,370 | 1,230 | 6 | 498 | 2,000 | 42.6 | 7.9 | 4.2 | Not done |
| 1/10/47 | 3:00 P.M. | 12,700 | 11,180 | 1,060 | 4 | 413 | 2,000 | 41.9 | 7.8 | 4.1 | 1/30 |
| 1/10/47 | 4:50 P.M. | 14,370 | 12,620 | 1,106 | 11 | 604 | 2,000 | 41.8 | 7.9 | 4.1 | 1/30 |
| 1/10/47 | 7:45 P.M. | 14,490 | 11,780 | 2,010 | 12 | 630 | 2,000 | 41.4 | 7.8 | 3.9 | 1/30 |
| 1/10/47 | 9:45 P.M. | 14,680 | 10,850 | 2,760 | 28 | 984 | 2,000 | 41.0 | 7.9 | 4.0 | Not done |
| 2/10/47 | 3:00 P.M. | 9,110 | Not done | Not done | 114 | Not done | Not done | 42.7 | 7.1 | 4.0 | Not done |

[1] ACTH (32D) ≡ 140 mg. Armour standard LA-1-A 55 mg. given at 9:45 A.M. 60 mg. at 11:00 A.M. and 85 mg. at 12 noon.

TABLE 5. *Experiment 4.* EFFECT OF 200 MG. ACTH ON THE BLOOD PICTURE OF A MALE SUBJECT (Ha)

| Date | Time | Leucocyte Counts Per Cubic Millimetre | | | | | Number of cells examined | Haematocrit Vol. % | Plasma proteins gms./100 ml. | Antihaemolysin PIU/ml. serum | Agglutinin Titre |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Neutrophil | Lymphocyte | Eosinophil (Direct) | Monocyte | | | | | |
| 18/11/47 | 10:00 A.M. | 6,350 | 3,683 | 1,600 | 117 | 871 | 2,000 | 47.8 | 7.4 | 4.9 | Not done |
| 18/11/47 | 2:00 P.M. | 6,500 | 3,650 | 1,960 | 140 | 754 | 2,000 | 44.6 | 7.1 | Not done | Not done |
| 18/11/47 | 6:00 P.M. | 6,310 | 3,430 | 1,944 | 110 | 769 | 2,000 | 44.6 | 7.1 | Not done | Not done |
| 19/11/47 | 10:00 A.M. | 9,410 | 6,630 | 1,657 | 139 | 979 | 1,000 | 47.2 | 7.3 | 7.0 | 1/20 |
| 20/11/47 | 10:00 A.M.[1] | 15,050 | 12,900 | 1,220 | 6 | 908 | 2,000 | 45.1 | 7.4 | 4.6 | 1/20 |
| 20/11/47 | 12 noon | 15,320 | 12,920 | 1,270 | 2 | 1,118 | 1,000 | 42.9 | 7.1 | 8.5 | Not done |
| 20/11/47 | 2:00 P.M. | 16,420 | 14,500 | 1,083 | 1 | 821 | 2,000 | 44.0 | 7.5 | 4.6 | Not done |
| 20/11/47 | 4:00 P.M. | 14,920 | 12,750 | 1,357 | 0 | 805 | 2,000 | 42.7 | 7.4 | 4.8 | 1/20 |
| 20/11/47 | 6:00 P.M. | 14,870 | 11,490 | 2,260 | 15 | 1,076 | 2,000 | 42.9 | 7.4 | 4.6 | 1/20 |
| 21/11/47 | 10:00 A.M. | 8,040 | 5,320 | 1,800 | 100 | 820 | 2,000 | 41.9 | 6.7 | 4.5 | 1/20 |
| 22/11/47 | 10:00 A.M. | 6,000 | 3,336 | 1,770 | 100 | 798 | 2,000 | 45.9 | 7.5 | 5.2 | 1/20 |
| 24/11/47 | 10:00 A.M. | 6,000 | 2,934 | 2,130 | 142 | 738 | 2,000 | 45.3 | 7.3 | 5.4 | 1/20 |

[1] ACTH (32D) ≡ 140 mg. Armour standard LA-1-A given in six equal doses. The first was injected at 2:05 P.M. on the 19th November and the remainder at four hourly intervals. The last injection was given immediately after withdrawal of this sample.

3) the titre increased at 3 hours from 1/80 to 1/160, but a change of this degree in agglutination titres is of doubtful validity. A similar change in the opposite direction was noted in experiment 1 (Table 2). In Figure 2 the values for 11-oxy steroids observed in experiment 3 are also charted. These were obtained for three six hour urine collections made between 10 A.M. and 4 P.M. on three consectuive days. In the six hours following the injection of corticotrophin there was a fivefold increase in the excretion of these compounds indicating that adrenal cortical activity was in fact increased. In the first two experiments, biological assay of the urine for three 48 hour periods was carried out and showed no change in the sample covering the experimental day. Presumably the cortical response was so brief that it did not noticeably change the total excretion of corticoids occurring in 48 hours.

*Experiments 4–5.* Having failed to observe any appreciable rise in antibody levels in the above experiments it seemed possible that a longer period of adrenal stimulation might be necessary to produce this effect.

In experiment 4 (Table 5) six injections at four hourly intervals were given, such that the total quantity of hormone received was 200 mgs. This preparation of the hormone was the same as that used in experiments 1 to 3. It caused slight dizziness and marked pallor in the subject, together with some abdominal cramps. The values obtained for the anti-haemolysin in this experiment are puzzling. Two control sera were obtained and a marked rise (43%) in the anti-haemolysin level was seen to have occurred before injection of the hormone started. No further blood samples were withdrawn until immediately before the last injection. The anti-haemolysin at this time had fallen to the initial control level. Two hours later it had risen 73% above this and after four hours had again returned to the previous level. At first it seemed that the samples drawn at 10 A.M. on the 19th and the 20th might have been inadvertently interchanged and that in fact a rise in antibody titre had been induced by administration of the hormone. There are three things which suggest that this was not a correct hypothesis. In the first place there was an interval of 24 hours between the taking of the two samples, making it extremely unlikely that mis-labelling had occurred. Secondly, if the anti-haemolysin level at 10 A.M. on the 20th was 7.0 units it must have been due to an effect lasting at least four hours, as the previous injection of corticotrophin had been given at 6 A.M. A similar effect should then have been noted not only in the 12 noon serum, which was taken two hours after the last injection, but also in the 2 P.M. sample. Finally, there was no parallel change in the typhoid agglutinin titres.

In view of these inconclusive findings the next experiment was designed along similar lines. By then a new preparation of cortico-

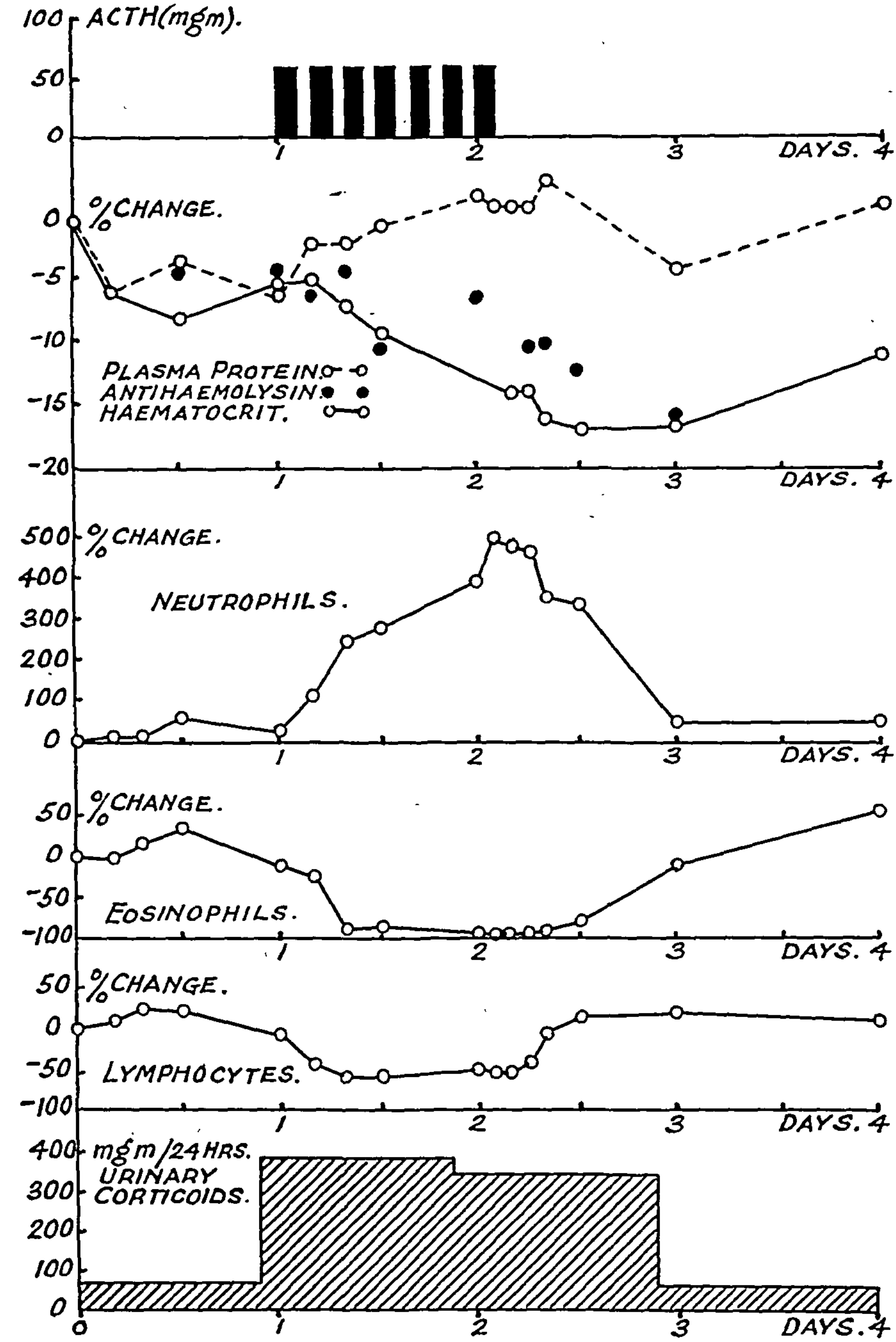


FIG. 3. Experiment 5. Subject H. The effect of 420 mg. ACTH (see Table 6).

trophin (Batch 37KE) had become available. This material had a very low pitressin activity and consequently a larger amount of the hormone could safely be injected at one time. In all 420 mgs. were

that used by Hartley and Smith (1935) for the international standardisation of staphylococcal antitoxin. It involves the use of a quantity of toxin some 50 to 100 times greater than that used by Chase, White and Dougherty. Recognising that the method used by these workers might be sensitive to smaller changes in antibody titre, the sera obtained in one of the above experiments was titrated at different toxin levels. No difference in the relative levels of antitoxin could be detected, whether the titration was made against 1, 1/5 or 1/50 L.H. unit. Difference of technique therefore cannot, in this instance, explain the difference in results. Another reason for the lack of effective change in antibody titres may be that hyperimmune subjects were not used. This idea is suggested by the findings of Eisen *et al.* They noted that the animals which did show rises in antibody nitrogen were those which had previously shown high titres after immunization. The course of staphylococcal toxoid given to the subjects of the present experiments was designed for routine therapeutic use and consequently is not comparable to the antigen stimulus usually given to experimental animals. While it is possible to reach levels around 40 PIUs by repeated courses, this was not attempted. Possibly antibodies are only stored in lymphocytes when larger quantities of antibody have been manufactured and these conditions were not attained in the above experiments. Finally, it cannot be assumed that a fall in circulating lymphocyte counts necessarily reflects a destruction of these cells throughout the body. The rapidity with which the counts return to normal would, if this were so, indicate an amazing capacity of the lymphoid tissue to produce these cells. One would expect to find large numbers of very immature cells appearing in the circulation, a phenomenon which was not noticed while making the counts.

## SUMMARY

Circulating antibody levels in normal human subjects were not increased following the administration of large quantities (40–400 mgs.) of adrenocorticotrophic hormone. The findings of other workers regarding blood eosinophil and lymphocyte counts have, however, been confirmed. Both types of cells showed diminished counts, the fall in eosinophils being more marked and of longer duration the larger the amount of hormone injected.

There was a rapid fall in the packed cell volume following the higher doses of corticotrophin which was maintained for many hours after the cell counts returned to normal. The increase in plasma volume was not accompanied by a lowered concentration of serum proteins.

## ACKNOWLEDGMENTS

We wish to thank Professor J. S. L. Browne at whose suggestion these experiments were initiated and Professor E. G. D. Murray,

both of whom have given us much help throughout the course of the work. Our thanks are also due to the experimental subjects for their very patient co-operation, and to Armour Laboratories Inc. for the gift of the hormone preparations used in this work. One of us (P.H.H.) is indebted to the Banting Research Foundation for a grant.

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# ADRENO-CORTICAL CHANGES IN SYRIAN HAMSTERS FOLLOWING GONADECTOMY

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IN THE course of gonadectomy studies with Syrian hamsters, changes in the suprarenal cortex were observed which may be of interest to other investigators. At the end of an experiment to test the effect of gonadectomy on dental caries in hamsters, autopsies were performed on all animals. A detailed account of the experimental procedures and other findings are available in previous reports (Keyes, 1946, 1948).

The weights of the suprarenal bodies are reported in Table 1. These data are in agreement with previous observations of Peczenik

TABLE 1. LITTER DISTRIBUTION OF ABSOLUTE AND RELATIVE SUPRARENAL WEIGHTS. VALUES IN MILLIGRAMS

| | Control Males | | Castrated Males | | Control Females | | Spayed Females | |
|---|---|---|---|---|---|---|---|---|
| Litter | Absolute Weight | mg/100 gm of body wt. | Absolute Weight | mg/100 gm of body wt. | Absolute Weight | mg/100 gm of body wt. | Absolute Weight | mg/100 gm of body wt. |
| B | 16 | 16.7 | 15* | 8.8 | 13 | 7.5 | 12 | 6.7 |
| C | 26 | 23.8 | 16 | 10.0 | 11 | 6.8 | 15 | 10.0 |
| D | 25 | 24.8 | 16 | 10.1 | 10 | 6.8 | 13 | 8.1 |
| E | 20 | 18.3 | 18 | 11.0 | 11 | 7.4 | 17 | 10.6 |
| G | 26 | 24.0 | 21 | 12.5 | 10 | 5.8 | 18 | 11.0 |
| H | 20 | 20.8 | 17 | 10.7 | 15* | 9.6 | 15* | 9.4 |
| I | 16 | 20.5 | 16 | 12.0 | 12* | 7.5 | 12 | 9.2 |
| K | 19 | 19.2 | 16 | 12.2 | | | 13* | 9.5 |
| L | | | 17 | 11.2 | 15 | 9.7 | 16 | 11.3 |
| M | 21* | 22.2 | 20* | 12.8 | 16 | 13.2 | 15 | 10.7 |
| M | 21 | 20.2 | | | 15 | 12.2 | | |
| Mean Wt. | 21 | 20.9 | 17 | 11.2 | 13 | 8.7 | 15 | 9.7 |

* Organ was slightly damaged in dissection. Values are not included in average weights, either absolute or relative.

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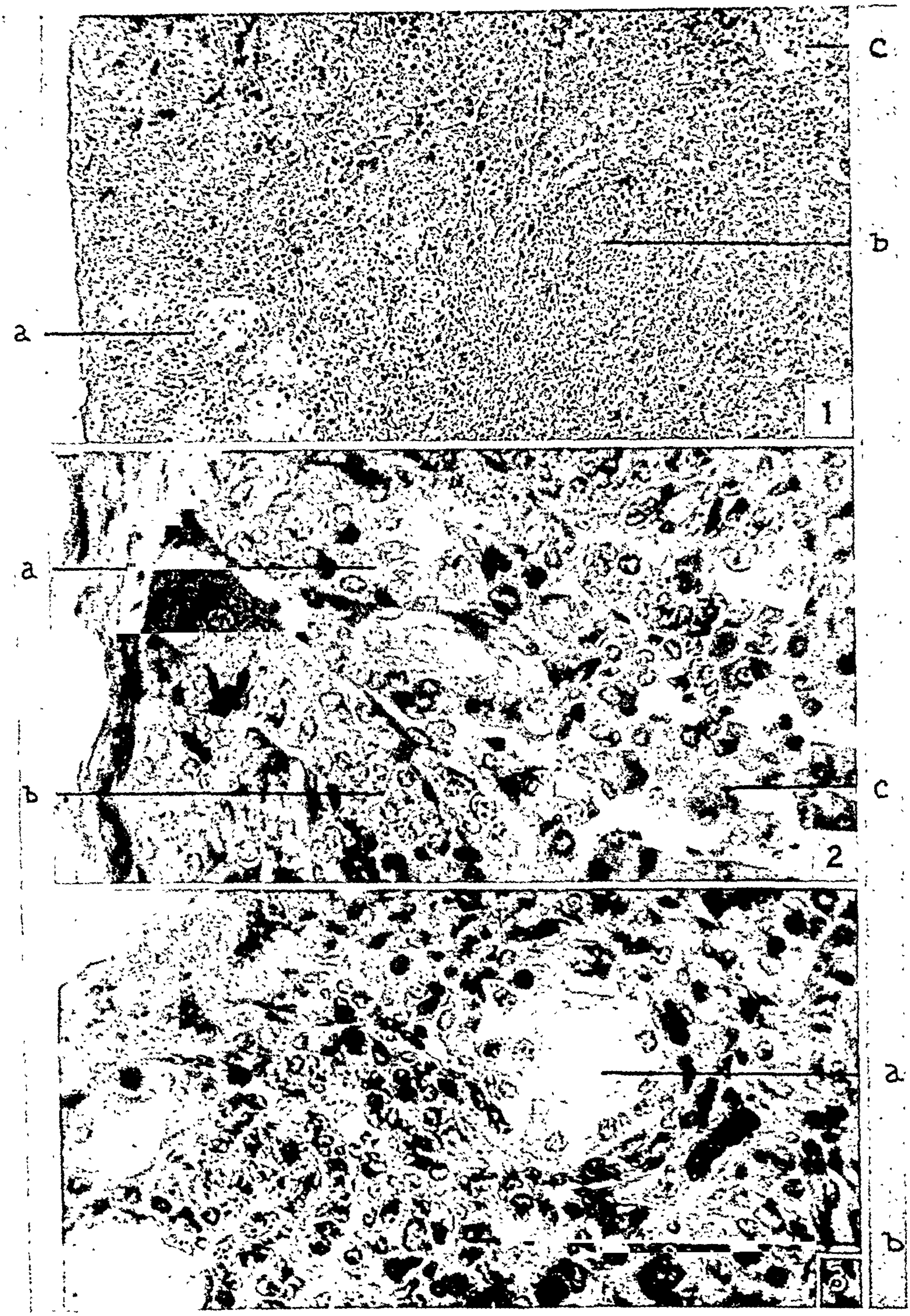
Sections of suprarenal cortex of gonadectomized hamsters. Zenker fixation; hematoxylin and eosin.

FIG. 1. a) Groups of enlarged cells in zona glomerulosa. b) Invasion of zona fasciculata by spindle-shaped cells. c) Medulla. (×100)

FIG. 2. a) Hypertrophied subcapsular cells in zona glomerulosa. b) Densely packed cells with indistinct cytoplasm and nuclei containing coarse chromatin material. c) Normal fasisculata cells. (×430)

FIG. 3. a) Follicle-like arrangement of large vesicular cells. b) Densely aggregated cells some of which appear to assume a spindle shape. (×430)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# QUANTITATIVE INTERFERENCE WITH ESTROGEN-INDUCED TISSUE GROWTH BY FOLIC ACID ANTAGONISTS[1]

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WE HAVE previously reported that the folic-acid deficient chick exhibits only a very limited response to even excessively high doses of administered estrogen (Hertz, 1945). A similar impairment in estrogen response has been observed in the folic-acid-deficient monkey (Hertz, 1948). Moreover, the degree of response to injected estrogen as evidenced by increase in weight of the genital tract in the chick was shown to be quantitatively related to the amount of folic acid ingested. These observations have been confirmed by Kline and Dorfman (1948).

Preliminary data have been previously presented indicating that the ingestion of a chemically unidentified, crude folic acid antagonist incorporated into a normal stock diet leads to a marked reduction in genital tract response to diethylstilbestrol in chicks (Hertz, 1948). Franklin et al. (1948) have reported similar observations.

The present report is concerned with further quantitative studies on the inhibition of estrogen-induced tissue growth in the genital tract of both the chick and rat by means of certain chemically identified folic acid antagonists.

## METHODS AND MATERIALS

(A) Chicks: New Hampshire Red chicks of the same flock were used throughout.[2] They were received the day after hatching and were maintained in electrically heated brooders. No food was given but tap water was provided ad libitum. On each of the first three days in the laboratory the chicks were injected subcutaneously with from 0.2 cc. to 1.0 cc. of an aqueous solution or suspension of the folic acid antagonist to be tested. In experiments designed to show the reversibility of the observed inhibition, the chicks also received subcutaneously each day 5 mg. of folic acid dissolved in 1 cc. of .05/N NaOH. The folic acid was always injected one hour before the administration of the antagonist. On each of the last two days of the test, 1 mg. of diethylstilbestrol dissolved in 0.2 cc. of corn oil was injected subcutaneously. Twenty-four hours after the last injection the animals were

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[1] Read before the annual meeting of the Association for the Study of Internal Secretions; June 19, 1948, Chicago, Ill.

[2] Obtained from Hall Bros. Hatchery, Wallingford, Conn.

autopsied. The body weight was recorded and the genital tract dissected out and weighed. Control groups of animals receiving diethylstilbestrol alone and those receiving no treatment were autopsied at the same time.

(B) Rats: Twenty-one day old weanling female rats of the Wistar Strain were ovariectomized and given a pulverized stock ration[3] supplemented with 2 per cent dried liver ad libitum. After a rest period of from 3 to 5 days each rat was given the test dose of folic acid antagonist subcutaneously in 0.2 cc. to 1 cc. of an aqueous solution or suspension on each of three consecutive days. On the second and third day of treatment the rats also received 10 micrograms of estradiol in 0.5 cc. distilled $H_2O$ subcutaneously. In the reversal experiments, the folic acid was administered subcutaneously in 1 cc. of .05/N NaOH at the rate of 5 mg. per day for 4 consecutive days, starting the day preceding treatment with the antagonist. At autopsy, 24 hours after the last injection, the uteri were dissected out, freed of distending fluid, and weighed to the nearest milligram.

The folic acid employed was crystalline pteroylglutamic acid. The antagonists investigated included:[4]

(I) 4-aminopteroylglutamic acid
(II) 4-aminopteroylaspartic acid
(III) 4-amino-$N^{10}$-methyl pteroylglutamic acid
(IV) 2,4-diamino-6,7-dimethyl pteridine
(V) 2-amino-4-hydroxy-6,7-di(p-amino-phenyl) pteridine
(VI) 2,4-diamino-6,7-diphenyl pteridine
(VII) 4-desoxypteroylglutamic acid
(VIII) 2-amino-4-hydroxy-6,7-diphenyl pteridine

## RESULTS AND DISCUSSION

Most of the detailed quantitative studies were done with 4-aminopteroylglutamic acid (I).

Figure 1 presents the data from a representative experimental chick series. It will be seen that doses of from 4 to 16 micrograms of 4-aminopteroylglutamic acid (I) effect progressive decrements in oviduct weight. This impairment in oviduct response is readily reversed by administration of an excess of folic acid.

Similar observations in the rat are summarized from 3 successive experiments in Fig. II. As in the chick, the significant features are (a) the distinct quantitative relationship between the inhibition obtained and the dose of antagonist administered and (b) the nearly complete reversibility of the inhibition by high doses of folic acid.

The observations made with the other antagonists on the chick are summarized in Table 1. It will be seen that 4-amino-$N^{10}$-methyl folic acid and 4-aminopteroylaspartic acid also exert an inhibitory effect which is reversible by folic acid in the former. A limited inhibitory effect is also observed with 4-desoxypteroyl-glutamic acid. The

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[3] Purina Chow.

[4] (I) (II) (III) and crystalline folic acid kindly supplied by the Lederle Laboratories, Pearl River, New York. (IV) (V) (VII) kindly supplied by Dr. R. A. Brown, Parke Davis & Co., Detroit, Michigan. (VIII) and (VI) kindly furnished by Dr. C. K. Cain, Cornell University, Ithaca, New York.

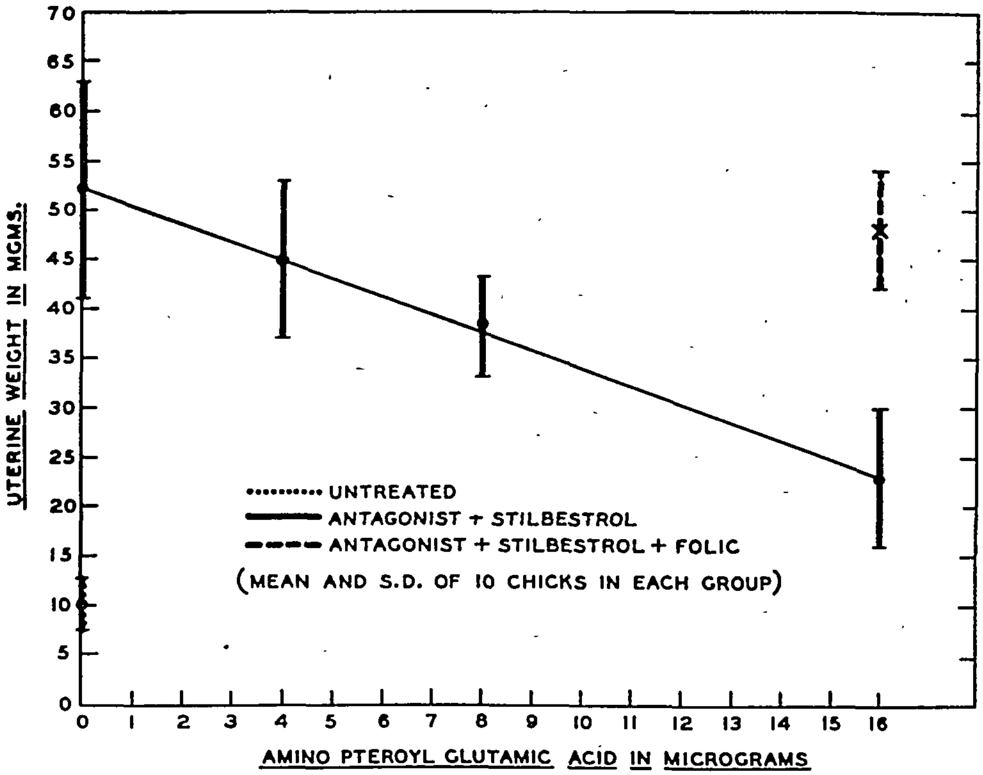


FIG. 1. Effect of folic antagonist on chick oviduct response to stilbestrol.

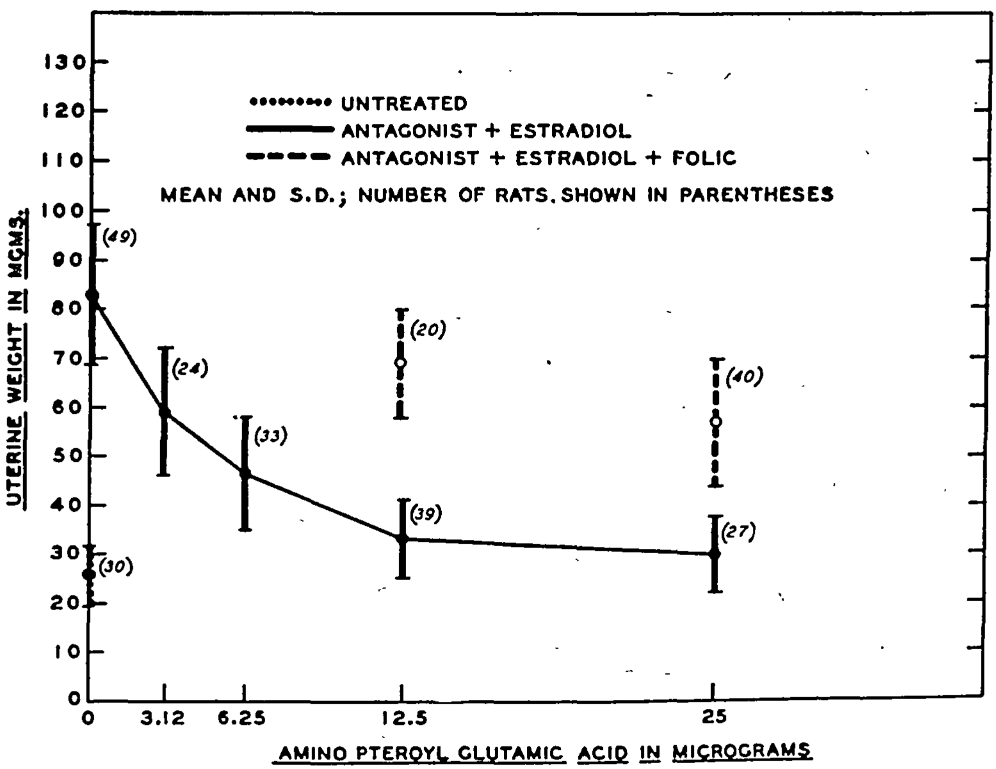


FIG. 2. Effect of folic antagonist on rat uterine response of estradiol.

pteridine compounds proved essentially ineffective at the moderate doses employed. These negative data serve to indicate the specificity of the inhibition obtained with the more active compounds.

The marked dependence of a specific tissue growth response upon a dietary trace factor is a phenomenon of both practical and theoretical interest. Further study of this and related effects may serve to elucidate the metabolic mechanisms involved in this type of hormonal response. In the case of folic acid there is not yet decisive information as to the precise metabolic role of this vital substance in cellular metabolism. There is some indication that the vitamin is concerned with the metabolism of thymine and other pyrimidines and purines (Stokes, 1944). However, when the essential function of folic acid becomes clarified, we may also gain further knowledge of some of the mechanisms involved in estrogen-induced tissue growth.

TABLE 1. EFFECT OF FOLIC ACID ANTAGONISTS ON ESTROGEN RESPONSE IN CHICK OVIDUCT[1]

| Antagonist | Daily Dose mg. | Oviduct Weight mg. |
|---|---|---|
| 4-amino-N$^{10}$-methyl pteroylglutamic acid | 0.2 | 23 ± 5 |
| | 1.0 | 23 ± 3 |
| | 0.2* | 57 ± 8 |
| | 0.0 | 51 ± 6 |
| 4-aminopteroylaspartic acid | 0.2 | 48 ± 5 |
| | 0.4 | 44 ± 5 |
| | 0.8 | 33 ± 9 |
| | 2.0 | 29 ± 6 |
| | 3.0 | 22 ± 7 |
| | 4.0 | 20 ± 3 |
| | 0.0 | 54 ± 8 |
| 4-desoxypteroylglutamic acid | 1.0 | 50 ± 7 |
| | 5.0 | 55 ± 6 |
| | 25.0 | 47 ± 6 |
| | 50.0 | 23 ± 4 |
| | 0.0 | 59 ± 5 |
| 2,4-diamino-6,7-dimethyl pteridine | 1.0 | 48 ± 5 |
| | 5.0 | 58 ± 12 |
| 2-amino-4-hydroxy-6,7-diphenyl pteridine | 1.0 | 50 ± 4 |
| | 5.0 | 53 ± 5 |
| | 9.0 | 47 ± 4 |
| 2-amino-4-hydroxy-6,7-di(p-aminophenyl) pteridine | 1.0 | 48 ± 6 |
| | 5.0 | 62 ± 11 |
| 2,4-diamino-6,7-diphenyl pteridine | 1.0 | 52 ± 4 |
| | 5.0 | 51 ± 8 |
| | 9.0 | 52 ± 9 |

[1] All animals treated as described in text; 10 chicks in each group.
* Also given 5 mg. folic acid daily.

The quantitative character of the inhibition obtained in both the

chick and the rat suggests an intimate and dynamic relationship between the hormone and the vitamin. It is noteworthy that this relationship applies to estrogens of both the stilbene and steroid type and can be demonstrated in both bird and mammal.

The available data do not warrant the conclusion that the interaction between folic acid and its antagonists in the quantitative determination of estrogen-induced tissue growth may be considered a strictly competitive relationship. Before such a conclusion may be reached there will be required data indicating the minimal amount of folic acid needed to reverse the inhibition produced by variable amounts of antagonist. The data at hand simply demonstrate that the expected level of tissue growth may be restored by an excess of folic acid.

It should be emphasized that the growth inhibition effected by the folic acid antagonists occurs in a tissue which is under the influence of a highly potent growth stimulus administered at enormous dose levels. Further study of the quantitative ratio between hormone dose and the quantity of antagonist required for maximum inhibition may serve to further characterize the vitamin-hormone relationship.

The interference with the action of a hormone by an anti-vitamin has certain implications for therapy in those conditions in which it may prove desirable to reduce the biological effectiveness of endogenous hormones, as in the case of prostatic carcinoma (Huggins et al. 1941); and cancer of the breast (Boyd, 1900). The present observations may serve as a basis for further exploration in this field.

## SUMMARY

Quantitative inhibition of estrogen-induced tissue growth in the female genital tract by a number of folic acid antagonists is demonstrated in the stilbestrol-treated chick and in the estradiol-treated ovariectomized rat. This inhibition is reversed by the administration of folic acid.

The significance of quantitative interference with hormonally induced tissue growth by an anti-vitamin is discussed.

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# THE ELEVATION OF PLASMA RIBOFLAVIN IN ESTROGEN TREATED FEMALE CHICKS

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A MARKED increase in the fluorimetrically determined riboflavin content of the serum of the immature female domestic fowl undergoing treatment with estradiol dipropionate was reported by Comman and Bolton (1946), and Common, Rutledge and Bolton (1947).

We have further investigated this phenomenon as a part of a more general study of the role of essential nutrients in hormonal stimulation of tissue growth. An increase in plasma riboflavin was observed in female chicks treated with diethylstilbestrol, the riboflavin being determined both fluorimetrically and microbiologically. In addition, we have attempted to determine the source of the elevated plasma riboflavin by analyses of muscle and liver of chicks maintained on a complete and on a riboflavin deficient diet.

It was also observed that the blood nicotinic acid content, in marked contrast with the riboflavin content, is not materially altered by estrogen administration.

## MATERIALS AND METHODS

New Hampshire Red female chicks from the same flock were used throughout.[1] These chicks were received the day after hatching and were placed immediately either on a stock diet,[2] or on a synthetic diet of known riboflavin content (Franklin *et al.*, 1947).

Estrogen treatment consisted of the subcutaneous administration of 1 mg. diethylstilbestrol in 0.2 cc. corn oil daily for 5 days. Control chicks received equivalent amounts of corn oil alone. Treatment was begun when the chicks were 15 to 20 days of age. Autopsy was performed 24 hours after the last injection in all instances, except in those chicks described in Table 2, which were retained for the indicated intervals after injection in order to determine the rate of decline in riboflavin after cessation of treatment.

Blood and tissue samples taken from individual birds were prepared for assay according to Loy (1947). For the microbiological assays for riboflavin and nicotinic acid, Lactobacillus casei was grown on the medium described by Teply and Elvehjem (1945) and the method of Snell and Strong (1939) was employed.

The fluorimetric determinations were made by the official method of the

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[1] Obtained from Hall Bros. Hatchery, Wallingford, Conn.

[2] Purina Startena.

A.O.A.C. (1945), except that the addition of 3 drops of concentrated NaOH was employed for the development of the blank, this procedure having proven adequate for the complete inactivation of the riboflavin present (Robertson and Kahler, 1942).

## RESULTS AND DISCUSSION

A marked elevation in plasma riboflavin following estrogen administration is seen in both normal and riboflavin deficient birds, although the rise is not as great in the deficient animal. Nevertheless, the liver and muscle content of riboflavin remained unaltered in both normal and deficient chicks receiving estrogen. The increase in plasma riboflavin can be induced by estrogen administration in the deficient bird even though the liver content of riboflavin is reduced to about half the normal level. The muscle content is unaffected either by the riboflavin deficiency or by estrogen treatment. The data therefore indicate that the serum elevation is not necessarily accompanied by a marked tissue depletion and that this increase in plasma level does not require full tissue saturation.

TABLE 1. EFFECT OF DIETHYLSTILBESTROL ON RIBOFLAVIN CONTENT OF CHICK LIVER, PLASMA, AND MUSCLE*

| Diet | Stilbestrol Treated | No. of Chicks | Assay Method[1] | Plasma | Liver | Muscle |
|---|---|---|---|---|---|---|
| Stock | + | 24 | M | 1.21 ± .45 | 14.8 ± 1.2 | 2.2 ± .1 |
| | | | F | .47 ± .20 | 15.5 ± 2.7 | 2.0 ± .3 |
| | − | 21 | M | 0.34 ± .08 | 13.9 ± 1.3 | 2.6 ± .1 |
| | | | F | .07 ± .04 | 14.4 ± 2.1 | 2.0 ± .2 |
| Riboflavin Deficient | + | 12 | M | 0.61 ± .19 | 7.8 ± 0.5 | 2.5 ± .4 |
| | | | F | .09 ± .03 | 7.8 ± 1.4 | 2.1 ± .4 |
| | − | 18 | M | 0.32 ± .08 | 8.7 ± 2.1 | 2.4 ± .3 |
| | | | F | .06 ± .08 | 9.0 ± 1.8 | 1.9 ± .2 |

* All values are expressed as micrograms of riboflavin per gm. of fresh tissue or cc. of fresh plasma.
[1] F = fluorimetric; M = microbiological.

The maintenance of an elevated plasma riboflavin level after cessation of estrogen injection continues for less than six days, (Table 2). These data suggest that the continued mobilization of the vitamin requires a relatively high hormone level.

The nicotinic acid content of the blood was determined largely for comparison with the riboflavin content. The data in Table 3 indicate that the blood content of nicotinic acid is not materially altered by estrogen administration. This observation serves to emphasize the significance and specificity of the riboflavin effect.

It will be noted (Table 1) that there is excellent agreement in the values obtained by fluorimetric and microbiological assay in the case of liver and muscle. However, the serum assays show a higher value for riboflavin by the microbiological method than by fluorimetric de-

TABLE 2. BLOOD RIBOFLAVIN AFTER DISCONTINUATION OF ESTROGEN

| No. of Days* | No. of Chicks | Assay[1] |
|---|---|---|
| 1 | 6 | 0.79 (.21 -1.7) |
| 6 | 4 | 0.23 (0.17-0.27) |
| 11 | 4 | 0.18 (0.15-0.21) |
| 15 | 4 | 0.22 (0.12-0.27) |
| 20 | 3 | 0.11 (0.08-0.13) |
| 25 | 4 | 0.16 ( .14- .18) |
| —** | 4 | 0.15 (0.12-0.18) |
| —*** | 4 | 0.12 ( .08- .15) |

* Number of days after last stilbestrol injection.
** Untreated Controls killed at beginning of experiment.
*** Untreated Controls killed at end of experiment.
[1] Microbiologically determined riboflavin in micrograms per cc. fresh blood; range in parentheses.

TABLE 3. EFFECT OF ESTROGEN ON BLOOD CONTENT OF NICOTINIC ACID

| Sample | Stilbestrol | Assay[1] |
|---|---|---|
| A | + | 8.0 |
| | — | 10.0 |
| B | + | 8.0 |
| | — | 10.5 |
| C | + | 10.9 |
| | — | 12.4 |
| D | + | 9.3 |
| | — | 12.7 |

[1] Expressed as micrograms of nicotinic acid per cc. fresh blood.

termination. This discrepancy is probably attributable to the presence in chick blood of non-specific lipid materials which stimulate the growth of L. casei, (Strong and Carpenter, 1942). It is pertinent that estrogen administration leads to a marked lipemia in the bird, (Fleischmann and Fried, 1945). Such a lipemia would presumably increase the blood content of the factors stimulating bacterial growth. Accordingly, further study of the hormonal factors involved in the

mobilization of these growth-promoting lipids and their potential effect upon the tissue growth response to estrogens in the female genital tract may prove fruitful. Moreover, our microbiological assays for riboflavin in plasma must be considered only as comparative rather than as absolute values.

## SUMMARY

Earlier reports of a marked increase in plasma riboflavin in the chick after estrogen administration have been confirmed. This elevation in plasma riboflavin titre was also observed in riboflavin deficient chicks. The muscle and liver content of riboflavin was not altered by estrogen administration in either normal or riboflavin deficient chicks. In contrast to the riboflavin effect, the nicotinic acid content of the blood remained unaltered after estrogen treatment. The blood riboflavin levels returned to normal within six days following cessation of estrogen injections.

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# NOTES AND COMMENTS

## INTERPRETATION OF EXPERIMENTAL RESULTS OBTAINED WITH DIBENAMINE[1]

Demonstration of the highly effective and specific adrenergic blocking action of Dibenamine (N,N-dibenzyl-$\beta$-chloroethylamine) (Nickerson and Goodman, 1945, 1947, 1948; Nickerson and Nomaguchi, 1948) suggested its use as a tool in determining the role of excitatory adrenergic stimuli (epinephrine or sympathetic nerve impulses) in a variety of physiological processes. Among the papers reporting the use of Dibenamine in this capacity are those of Sawyer *et al.* (1947, 1948) on the prevention of post-copulatory ovulation in the rabbit by Dibenamine, and that of Rockwell (1948) on the therapeutic effect of Dibenamine in certain psychoses. In both cases, the conclusion was reached that blockade of adrenergic stimuli was causally related to the effects observed after the administration of Dibenamine. A careful analysis of the pharmacology of this drug indicates that alternative explanations must be considered.

Although the adrenergic blocking action of Dibenamine (and related $\beta$-haloalkylamines) usually overshadows all other actions of these compounds, it must be kept in mind that no known drug is absolutely specific. The following points are of particular importance in evaluating the results of experiments employing Dibenamine.

1. Dibenamine has a direct excitant action on the central nervous system. This effect appears to be independent of the adrenergic blocking action. It occurs earlier and is of much shorter duration than the blockade. Central nervous system excitation is particularly prominent after rapid intravenous administration of the drug and under these circumstances may be the most prominent action.

2. Dibenamine has been shown to block primarily the excitant effects of adrenergic stimuli on smooth muscle and exocrine gland cells. It has not been shown to block any adrenergic metabolic effects or the actions of adrenergic stimulation on the central nervous system, such as hyperventilation, analepsis, etc.

3. In contrast to the direct central stimulating action of Dibenamine, the adrenergic blocking action develops slowly. This action appears earlier when large doses of the drug are employed, but present results indicate that maximum blockade does not occur until at least $1\frac{1}{2}$ hours after intravenous administration.

All these points apply to an interpretation of the work of Sawyer and associates. The rapid injection and short time-intervals involved in their experiments at once suggest the possibility that the direct effect of Dibenamine on the central nervous system rather than its adrenergic blocking

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[1] Dibenamine and 2-dibenzylaminoethanol were kindly supplied by Dr. William Gump of Givaudan-Delawanna, Inc.

action may have been the critical factor in preventing ovulation. (The injections in their experiments were regularly followed by signs of extreme central nervous system stimulation.) In the work of Rockwell, cerebral vascular changes as well as direct central nervous system stimulation must be considered.

Fortunately, the above difficulties of interpretation can be largely overcome by control experiments employing 2-dibenzylaminoethanol. This compound is the hydrolysis product of Dibenamine in which the β-chlorine is replaced by an hydroxyl group. It has no demonstrable adrenergic blocking or local necrotizing action, but retains most of the other pharmacological properties of Dibenamine. Its direct action as a central nervous system stimulant in comparison with that of Dibenamine is shown in Table 1. The slightly

TABLE 1. COMPARISON OF CENTRAL NERVOUS SYSTEM STIMULATION PRODUCED BY DIBENAMINE AND 2-DIBENZYLAMINOETHANOL

| Dose[1] mg./kg. | No. of Mice | Convulsions % | Ave. Duration of Convulsions[2] min. | Mortality[3] % |
|---|---|---|---|---|
| | DIBENAMINE | | | |
| 25 | 10 | 80 | 0.29 ±0.07 | 0 |
| 35 | 10 | 100 | 2.8 ±0.60 | 0 |
| 50 | 10 | 100 | 7.9 ±1.7 | 40 |
| | 2-DIBENZYLAMINOETHANOL | | | |
| 18 | 10 | 60 | 0.66 ±0.25 | 0 |
| 25 | 15 | 100 | 2.2 ±0.46 | 40 |
| 35 | 15 | 100 | 4.0 ±0.53 | 53 |

[1] All injections had a volume of 5–10 ml./kg. and were made in 5 seconds.
[2] In animals which survived.
[3] All deaths were due to asphyxia during convulsions.

greater potency of 2-dibenzylaminoethanol and its somewhat greater ratio of lethality to duration of convulsions are probably attributable to its greater aqueous solubility. The compound also produces an increased respiratory exchange and other signs of central nervous system stimulation.

It may be hoped that the employment of 2-dibenzylaminoethanol as a control substance in experiments involving the adrenergic blocking action of Dibenamine will materially clarify interpretations of the results obtained.

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# MALE MATING BEHAVIOUR SHOWN BY FEMALE RATS TREATED WITH ENORMOUS DOSES OF ESTRONE

Stone 1924 and Beach 1938 reported that in male rats, which were vigorous copulators, a tendency to develop certain elements of female mating behaviour could be observed. Later, Beach (1941) demonstrated that large doses of testosterone-propionate elicit female mating behavior in male rats, especially if these animals are excited by a previous copulation which was interrupted before ejaculation.

We have shown (Engel, 1942) that similar effects can be produced in male mice by testosterone propionate as well as by estradiol benzoate or by yohimbine. We affirmed Beach's theory that in those cases the reactions are rather due to a sexual (ambivalent) excitation of the nervous system, which is capable of producing the mating pattern of either sex. Recently we have extended this theory by observations in female rats, where also sex hormones elicit an apparently homosexual or lesbic reaction. Beach (1942) had shown that testosterone may produce male mating behaviour in spayed female rats.

Two groups of rats, one of four adult female rats, which had been spayed one year ago being then very young, virginal animals, and a second group of three adult normal female rats aged one year, received each one a deposit of 10 milligrams (100,000 I.U.) of estrone in form of microcrystals by intramuscular injection into the right hind leg.

From the second day and during a long period following, the animals of both groups were in perfect estrus. In order to determine the permanence of this state, vaginal smears were taken. From the tenth day we observed that after taking the vaginal smears (which was done with a small piece of cotton fixed on a very fine pincette), all animals were in a state of sexual hyperexcitation. It should be mentioned that the spayed animals had never had any contact with a male, and the normal females had not had such intercourse for some months. In the present experiment we observed that the excited animals began to chase each other, in the same manner a male rat would do, licking the genital region and afterwards trying to mount.

Normal, or spayed females, excited only by friction of the vagina, never showed a similar reaction in presence of other female rats, whilst each of the rats treated with 100,000 I.U. of estrone reacted as noted above. There was no difference between spayed and normal female rats; the animals not only attacked rats in permanent estrus, but also proestric, or metestric females, as well as spayed rats. But, as there was no reaction on the side of the attacked animals, the pursuers quickly ceased to be interested in them.

This indicates a certain difference between the behaviour of the normal male, which will attack only estrous females, and treated animals. It agrees very well with Beach's observation that young male rats, made "hyperexcitable" by testosterone-propionate, displayed a tendency to mate with non-receptive female rats, male rats, and even guinea pigs.

Once more we come to the conclusion that a very strong hormonal hyperexcitation elicits the nervous reactions in sexual behavior to attack whatever object; but this is rather to be considered as a general hypersexuality than as a transformation into a homosexual attitude.

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SUMMARY

Female rats, entire as well as spayed, injected with 10 mgms. of estrone in microcrystals, excited by friction in taking vaginal smears, attack other females of their species, chasing and even mounting them, showing male mating behaviour.

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# ASSOCIATION NOTICE

## ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

## POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

# ASSOCIATION AWARDS FOR 1949

## THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

## THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

## THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

ENDOCRINOLOGY

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# QUANTITATIVE STUDIES OF CELL TYPES IN THE RAT HYPOPHYSIS FOLLOWING UNILATERAL ADRENALECTOMY

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THE INTERRELATIONSHIP between hypophysis and adrenal cortex has been established conclusively by extensive experimentation with hypophysectomized animals, through the use of pituitary implants and by means of injections of extracts into both intact and hypophysectomized animals. Reduction of adrenal cortical hormone concentration in the blood is believed to increase hypophyseal adrenotrophic secretion, resulting in hypertrophy and hypersecretion of the adrenal cortex (Tepperman, Engel and Long, 1943). Nevertheless, in spite of remarkable agreement among present investigators concerning the relationship between pituitary gland and adrenal cortex, there is lack of agreement regarding the specific type of cell in the anterior hypophysis which is responsible for secretion of the adrenocorticotrophic hormone. Until recently, most studies of this problem had been limited to observations on the histology of the pituitary gland following death from Addison's disease (Kraus, 1927), or in the terminal stages of complete adrenalectomy of experimental animals. The most common findings under these conditions have been diminution and degeneration of both acidophiles and basophiles. Crooke and Russell (1935) state that the constant features in pathological adrenal insufficiency are the extreme decline in the number of basophile cells, a variable reduction in the number of acidophiles, the presence of a series of abnormal basophilic cells and of a considerable number of large chromophobes. Severinghaus (1938) describes one case of a woman with Addison's disease in which there was a marked increase in basophiles. In summarizing the results in this field, Severinghaus states, "Addison's disease in general seems to be associated with a depletion of basophiles from the hypophysis, but certain glands have

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[1] Heermans Fellow in Anatomy 1946–47.

appeared to show an attempt to attain normalcy, a process which may be of such short duration as to be generally missed. Basophilic activity seems indicated." Reese, Koneff and Akimoto (1939), in studying the anterior pituitary glands of male rats showing acute symptoms after double adrenal ablation, find diminution in number and size of acidophiles, correlated with progressive loss of granular material and regressive changes in the Golgi apparatus. The basophiles of their rats are also obviously diminished in number and size with cytological changes being dependent upon severity of symptoms and length of post-operative period. The observations, upon which these conclusions are based, were on terminal stages of bilateral adrenalectomy in experimental animals, or on cases of Addison's disease; thus the question arises as to whether the changes were a result of modification in pituitary-adrenal cortical relationship, or of abnormal metabolism of the body as a whole (inanition?).

Heinbecker and Rolf (1944) attribute adrenotrophic secretion to the eosinophilic cells: they find functional depression of the adrenal cortex followed by progressive atrophy in hypophysectomized dogs, whereas when the infundibular stem is severed and fibers from the paraventricular nucleus are cut by a puncture wound in the posterior hypothalamus the eosinophile cells are maintained, the basophiles decrease in number and the adrenal cortex remains normal.

D'Angelo, Gordon and Charipper, (1948) have utilized the characteristic absolute adrenal hypertrophy of guinea pigs following deprivation of food as a means of determining the cellular basis for adrenocorticotrophic secretion. Under these conditions of probable corticotrophic hypersecretion they find progressive loss of acidophilia, and an increase in number of basophiles and chromophobes. "These results are interpreted to mean that cortical hypertrophy in the starving guinea pig results from augmentation of adrenotrophin secretion by the basophiles of the anterior pituitary." Unfortunately, the cortical hypertrophy of inanition and the cellular changes in the hypophysis have not been produced in animals other than guinea pigs, and the cortical hypertrophy seems to be unaffected by exogenous cortical hormones.

The present observations are on unilaterally adrenalectomized rats, in which relative reduction in adrenal cortical tissue stimulated adrenocorticotrophic activity so that compensatory hypertrophy of the remaining adrenal gland occurred. These animals were healthy, well-nourished and continued to grow so that their body weights were only slightly lower than those of unoperated control rats.

## PROCEDURE

The results of this investigation are based on quantitative histological studies of the pituitary and adrenal glands of one hundred and twenty male rats of the Sprague-Dawley strain. Male rats were used to avoid the compli-

cations of any cyclic phenomena associated with the estrous cycle. The left adrenal glands were removed from all experimental animals at the age of 31 days. One group was kept at laboratory temperature (Approx. 26°C.), and a second group was placed in a cold room kept at 5.6°C. to apply an additional stimulus to compensatory hypertrophy. Autopsies were performed 5, 10, 15, and 25 days following operation. The hypophysis was removed and immediately fixed in Zenker-formol solution. Adrenal glands, thyroid gland, thymus and testes were weighed on a torsion balance and fixed in 10% formalin.

The pituitary glands were sectioned in a horizontal plane at 4 micra and stained with a modified azocarmine stain (Briseno-Castrejon and Finerty, 1949). In this method the nuclei were pre-stained by a short immersion in alum hematoxylin. This was followed by mordanting in aniline alcohol and a 45 minute period in azocarmine solution at 60°C. The counterstains, acid green and orange G, were dissolved in clove oil to avoid destaining of the azocarmine. After the above staining, acidophilic granules are purplish red; basophile granules are light green; nuclear membranes are sharply defined; mitochondria are orange-red; red blood cells are brilliant orange; Golgi apparatus shows as a negative image in both types of chromophiles; and chromophobes show little or no cytoplasm, which is colorless to pale green.

Quantitative determinations of the percentages of each cell type were made by differentially counting all the cells in every fifth field of three horizontal sections at equidistant levels in each gland. Approximately 3000 cells were counted in each gland.

## RESULTS AND DISCUSSION

The consistent responses to unilateral adrenalectomy are compensatory hypertrophy of the remaining adrenal gland and increased percentage of acidophiles in the anterior hypophysis, as is shown in the summarized results in Table 1.

In all cases of unilateral adrenalectomy, compensatory hypertrophy of the remaining adrenal gland occurred. Using the mean weight of the right adrenal glands of the unoperated control rats as a base line, the percentage of absolute hypertrophy was calculated. There seems to be no consistent pattern of progressive hypertrophy, except that those rats exposed to the additional stress of a continual cold environment exhibit considerably more adrenal growth. When the basis of proportion of adrenal weight to body weight is considered, the hypertrophy is of a greater degree, especially in the cold-exposed rats, in which there is a greater absolute hypertrophy of the adrenal gland, which is accompanied by a lower body weight than in those kept at room temperature.

Since the adrenal glands appear to respond to the administration of various forms of stress only through the mediation of pituitary adrenotrophic activity (Sayers, Sayers and Woodbury, 1948) it may be assumed that the compensatory hypertrophy is a result of increased pituitary secretion. This assumption is substantiated by the changes which are shown in the percentages of cell types in the anterior hypophysis. In those rats which have been subjected to relative reduction

of circulating adrenal cortical hormones and are undergoing heightened adrenal stimulation, there is a significant increase in the percentage of acidophilic cells. Superficial examination of the hypophysis in unilaterally adrenalectomized rats in some cases conveys the impression that the number of basophiles has increased. However, detailed quantitative analysis demonstrates that there is an unequivocal augmentation in number of acidophiles balanced by a corresponding decrease in chromophobes. The apparent increase in percentage of

TABLE 1. THE EFFECT OF UNILATERAL ADRENALECTOMY AND EXPOSURE TO LOW TEMPERATURE ON THE PERCENTAGE OF CELL TYPES IN THE PITUITARY GLANDS OF IMMATURE MALE RATS

| | Days After Operation | No. of Rats | Rt. Adrenal Wt. (mg.) | Adrenal Hypertrophy[1] % | Pituitary Cell Type Analysis % Acid | % Baso | % Chrom. |
|---|---|---|---|---|---|---|---|
| Control | | 5 | 12.1 | | 41.7 | 7.6 | 50.3 |
| Operated (26°C.) | 5 | 5 | 15.5 | 28 | 47.4 | 7.3 | 45.3 |
| Operated (5.6°C.) | | 5 | 19.6 | 62 | 47.0 | 6.8 | 46.2 |
| Control | | 5 | 15.0 | | 39.7 | 8.0 | 52.3 |
| Operated (26°C.) | 10 | 5 | 20.0 | 33 | 49.9 | 8.4 | 41.7 |
| Operated (5.6°C.) | | 5 | 21.1 | 41 | 51.8 | 6.7 | 41.5 |
| Control | | 25 | 16.4 | | 37.4 | 7.5 | 55.3 |
| Operated (26°C.) | 15 | 25 | 19.8 | 21 | 49.2 | 7.5 | 43.3 |
| Operated (5.6°C.) | | 25 | 21.7 | 32 | 49.6 | 6.8 | 43.6 |
| Control | | 5 | 16.1 | | 37.9 | 7.4 | 54.7 |
| Operated (26°C.) | 25 | 5 | 19.7 | 22 | 53.7 | 7.8 | 38.5 |
| Operated (5.6°C.) | | 5 | 26.2 | 63 | 51.6 | 8.1 | 40.3 |

[1] The percentage of hypertrophy is calculated by using the mean weight of the right adrenal glands of the control rats as the basis for comparison.

basophiles in certain instances is due to their greater variability and to possible decrease in size of individual acidophiles. Statistical analysis of the percentages of acidophiles in the twenty-five control rats of the group studied fifteen days following operation shows a mean percentage of $37.4 \pm .52$.[2] Similar analysis of the twenty-five unilaterally adrenalectomized rats kept at room temperature shows a mean of 49.2 per cent $\pm .78$. The twenty-five unilaterally adrenalectomized rats kept at 5.6° C. show a mean of 49.6 per cent $\pm .61$. It appears that at 5 days following removal of one adrenal gland the changes in pituitary cell types have not reached a maximum, but that there is little progressive change after the 10 day period. The higher percentage in the 25 day group is difficult to interpret but is consistent in that none of the rats in this series had an acidophile count below 50 per cent. Experiments are now in progress to determine if, and when, the pituitary cytologic pattern returns to a normal level following the restoration of adrenal cortical function by the hypertrophied gland.

[2] Standard error.

A graphic presentation of the variability of acidophile cell percentages to demonstrate better the consistency of the data is shown in Figure 1. The 15 day series is shown in which there were 25 rats in each of the three groups. The rats are classified into four-percentile groups: all those with acidophile percentages between 32–35.9; 36–39.9; 40–43.9; etc. are placed in separate columns. It can be seen that the distribution of the control rat percentages (black columns) is

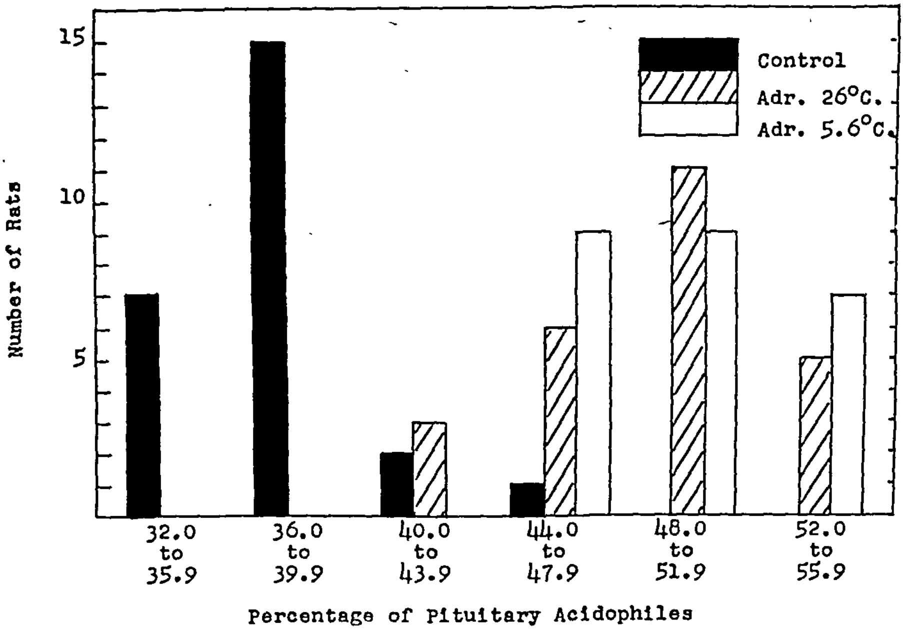


FIG. 1. Histogram to demonstrate graphically the frequency distribution of the percentages of acidophilic cells in the pituitary glands of untreated 46 day old male rats (black columns); 46 day old male rats which had been unilaterally adrenalectomized 15 days previously and kept at laboratory temperature (hatched columns); and 46 day old male rats which had been unilaterally adrenalectomized 15 days previously and kept in a cold room at 5.6°C. (white columns). The abscissa is subdivided into six successive groups of 4 per cent increments, into which are projected the number of rats having an acidophile count within each group. Note that the control rats show the greatest frequency between 36.0 and 39.9 per cent, whereas the adrenalectomized groups cluster about the 48.0 to 51.9 percentage group.

concentrated around the 36–39.9 group; the percentages with greatest frequency in the experimental groups are in the 48–51.9 group.

The basophile cells are the least numerous, ranging from 5.7 to 9.3 per cent in the control animals; from 5.1 to 9.2 per cent in the experimental rats at room temperature; and from 5.3 to 9.7 per cent in the experimental rats exposed to cold. The mean percentages in each group, however, show no effect of the adrenalectomy on the basophiles (Table 1).

No weight or volumetric estimations of the hypophysis were made at autopsy so as to avoid post mortem changes before fixation.

Micrometric measurements of every tenth serial section, however, demonstrate that the hypophyses of the rats exposed to cold were larger than those of animals (control, or adrenalectomized) kept at room temperature. This change was in the anterior lobe, since the pars intermedia and pars nervosa maintained a uniform size. This is in accordance with the observations made by Baillif (1938). Histological observations of the hypophyses of rats exposed to cold also confirm his findings that enlargement of the anterior lobe is due to increase in size of blood sinusoids, more numerous colloid vesicles and possible increase in size of basophiles. Extreme disintegration of the anterior lobe observed by Baillif (1938) under severe exposure to cold was not noted under the present experimental conditions, nor was cellular disintegration evident. Although no specific counts of mitotic index were made, more mitoses seemed to be present in the rats exposed to cold.

The weight of the thymus and of the testes was slightly reduced in the rats of the experimental groups, but when the weights of these organs is considered in relation to body weight it appears to be dependent upon the weight of the animal rather than upon the experimental procedures. The weight of the thyroid gland was unchanged which may indicate a slight hypertrophy in the experimental groups.

Much of the current knowledge concerning histological changes in the anterior lobe of the hypophysis produced by alteration in relationship between hypophysis and adrenal cortex is based on studies of the pituitary gland in Addison's disease, in bilaterally adrenalectomized animals, and to some extent in acromegaly. The sustentative data which are possible under controlled conditions of unilateral adrenalectomy have been ignored. In the histopathology of Addison's disease there is almost unanimous agreement in regarding the basophiles as the characteristic cells involved, even though the same authors describe similar alterations in the acidophiles; viz., a decrease in number of cells, indistinct and irregular cell borders, sparse cytoplasmic granulation and pyknotic nuclei. Histological evidence derived from the study of the anterior lobe after complete ablation of the adrenals has also produced contradictory and confusing results. Shumaker and Firor (1934) in describing the pars distalis of a bilaterally adrenalectomized dog, 128 days after operation, concluded that basophilic alterations, similar to those found in Addison's disease, are characteristic after complete removal of suprarenal glands. The results of Reese, Koneff and Akimoto (1939) also agree with this conclusion. Acromegaly is associated with hyperfunction of the pituitary gland and is characterized by acidophilic hyperplasia, or adenoma, accompanied by hyperplasia of the adrenal cortex. If the acidophilic hyperactivity brings about hyperplasia of the suprarenal tissue, this condition may be indicative that the adrenocorticotrophic principle is secreted by the acidophiles.

Thus, we have two opposite interpretations of the source of adrenocorticotrophin, obtained by different methods. From clinical observations in Addison's disease, the basophiles seem to be most involved (although they are described as showing degenerative effects); in acromegaly there is adrenal enlargement associated with acidophile hyperplasia. Experimentally bilateral adrenalectomy evokes degenerative changes in acidophiles and basophiles, whereas unilateral adrenalectomy produces acidophile predominance. The findings in acromegaly, as in Addison's disease or in bilateral adrenalectomy, may be the result of severe metabolic imbalance. Another possibility to explain these divergent findings, however, is that the presence of some normally functioning adrenal tissue is required for proper functioning of pituitary-adrenal interaction.

Since increased adrenotrophic activity, as indicated by adrenal hypertrophy, results from diminished adrenal cortical tissue, it has been suggested that reduction in circulating adrenal cortical hormone is the stimulus for pituitary adrenotrophic activity. Sayers and Sayers (1947) subjected adult male rats to various forms of stress for one hour and found that each type of stress produced an increase in adrenocorticotrophic activity, as determined by reduction in ascorbic acid content of the adrenal glands. Injection of appropriate amounts of cortical hormone prevented this hypersecretion. They conclude that, "the anterior pituitary elaborates adrenocorticotrophic hormone at a rate inversely proportional to the concentration of cortical hormone(s) in the body fluids and according to the requirements of the peripheral tissue cells for cortical steroids."

The results of the present experiments are interpreted to indicate that the increased adrenotrophic activity which follows reduction in circulating adrenal cortical hormone(s) is mediated by an increase in the number and activity of the acidophilic cells of the hypophysis, and that the source of adrenocorticotrophin is the acidophiles.

## SUMMARY

Unilateral adrenalectomy of immature male rats results in compensatory hypertrophy of the remaining adrenal gland and a marked increase in percentage of acidophilic cells in the anterior hypophysis. When unilaterally adrenalectomized rats are subjected to lowered environmental temperatures these changes are accentuated. It is believed that reduction in circulating adrenal hormone, either by removal of adrenal tissue, or by stress, stimulates an increase in number of pituitary acidophiles, and that these cells secrete adrenocorticotrophic hormone.

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# THE EFFECT OF HYPNOTICS ON BLOOD SUGAR AND ON THE ACTION OF INSULIN[1]

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THE EFFECT of barbiturates or morphine upon the blood sugar level has been the subject of several investigations but an examination of the results reported does not lead to convincing conclusions as will be evident from the following brief résumé.

Barbital sodium, as reported by Ellis and Barlow (1924), lowered the blood sugar in both cats and pigeons. Weiss (1926) has reported that barbital caused a marked initial rise in blood sugar in the cat and dog which is followed by a gradual fall. Jackson (1931) reported that in rabbits, barbital sodium retards the hypoglycemia produced by insulin. Murphy and Young (1932) have reported that pentobarbital and other barbiturates produced an increase in blood sugar in cats. Jarman and Abel (1933) reported that evipal sodium in humans produced no alteration in blood sugar. Hemingway et al. (1934) found that pentobarbital in the dog caused a slight increase in blood sugar. Goodman and Gilman (1941a) state "The response of the blood sugar to medication with the barbiturates varies considerably." La Barre and Vesselovsky (1942) reported that barbital sodium in dogs caused a fleeting rise in blood sugar.

Morphine, according to Ross (1918), caused a marked hyperglycemia in the dog. Stewart and Rogoff (1922a) report that morphine causes a marked hyperglycemia in the cat with lesser effects in the rabbit and dog. Bodo et al. (1938) reported that in adrenal-inactivated dogs and cats, morphine liberates sympathin which causes a slight hyperglycemia. Goodman and Gilman (1941b) state "The hyperglycemia noted after morphine in animals is inconstant." Meperidine hydrochloride causes a slight increase in the blood sugar of rabbits (Barlow, 1947).

Due to the lack of harmony of these reports, further investigation of the effect of these drugs on the blood sugar level is suggested. Since few of the previous investigators have been concerned with any effect of these drugs on the hypoglycemic action of insulin, this phase will

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be included in the present study. Since albino rats have a relatively constant blood sugar (Cole and Harned, 1938), they were chosen as the experimental animals.

## METHODS

Albino male rats, of the Wistar strain varying in weight from 150 to 350 grams, free from gross evidence of disease, were maintained on a commercial food (considered complete) supplemented by daily feeding with milk, whole wheat bread and lettuce. These animals also were given a few drops of fish oil[3] about every ten days. All food was removed from the cages 15–17 hours before each test began. In order to facilitate the securing of blood samples, the animals were placed in individual cylindrical wire cages with the tails protruding. The animals during the experimental procedures were kept at the temperature designated in Tables 1, 2, 3 & 4. Blood samples were obtained by amputating a thin segment of the tail with sharp scissors and then gently stroking the tail between the fingers, the blood being drawn into a glass capsule containing potassium oxalate. The blood sugar values were determined, after deproteinization (Somogyi, 1930), by either the method of Shaffer and Somogyi (1933) or Somogyi (1937). After taking the first sample which is designated "control," the drugs were injected and four more samples were obtained at successive one-hour intervals. The insulin injections were made subcutaneously and the other drugs were injected intraperitoneally. All injections were made aseptically. The doses mentioned are either units (U.) or milligrams (mg.) of the drugs per kilogram (kg.) of body weight. No animal was used without an interval of at least ten days elapsing between individual experiments.

## RESULTS

From the average control blood sugars presented in Tables 1, 3 and 4, it is evident that these values are relatively constant. There is also no significant change in the average blood sugar level in the normal groups over a period of five hours.

Morphine sulfate, at a low dose level (12 mg. Table 1, Group B) had no effect on the blood sugar level except a questionable slight increase at the end of hour 2. With the 25 mg. dose (Table 1, Group C) the average blood sugar level at the end of one hour is normal, but there were marked variations in the individual values. Since hyperglycemia[4] occurred in only 1 case in the 2, 3 or 4 hour blood sample with several cases of hypoglycemia,[5] the averages are below normal. In the 50 mg. group (Table 1, Group D) the highest recorded blood sugar average (82.2) was reached at the end of one hour as a result of 4 animals showing a hyperglycemia despite the fact that 5 animals

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[3] Vitamin A and D content equal to Cod Liver Oil U.S.P. XIII.

[4] Individual blood sugar values above 95 mg. per 100 cc. are considered hyperglycemic because the methods used show only "true sugar" and also the animals have been fasted for 15–17 hours which produced normal values of an average range from 63.7–71.5 mg. per 100 cc.

[5] Individual blood sugar values below 45 mg. per 100 cc. are considered hypoglycemic.

TABLE 1. THE EFFECTS OF INSULIN ON BLOOD SUGAR AS INFLUENCED BY HYPNOTICS

The average blood sugar values are expressed as mg. per 100 cc. The number of hyperglycemias are designated + and the hypoglycemias −. The animals were kept in a box maintained at 29°C. ±1° during the experimental procedure. The drugs were injected just after the control sample was obtained. The number of animals that died from this dose before the one hour sample was obtained is designated thus *. These animals were replaced to complete a group of ten in each case.

| Group | Experimental Condition | Control | Hour 1 | Hour 2 | Hour 3 | Hour 4 | Addenda |
|---|---|---|---|---|---|---|---|
| A | Normal Group | 67.8 | 65.8 | 67.6 | 68.2 | 62.4 | |
| B | Morphine Sulfate 12 mg. | 70.5 | 73.4 | 81.5 | 74.8 | 69.1 | |
| C | Morphine Sulfate 25 mg. | 64.6 | 65.0 | 59.9 | 60.4 | 57.9 | *2, +4, −11, 1 death each during hr. 1, 2 & 3 |
| D | Morphine Sulfate 50 mg. | 67.8 | 82.2 | 60.5 | 69.0 | 68.1 | *1, +7, −9, 1 death during hr. 1 & 3 during hr. 2 |
| E | Barbital Sodium 100 mg. | 67.9 | 64.3 | 57.9 | 60.8 | 60.4 | −1 |
| F | Barbital Sodium 200 mg. | 71.2 | 76.1 | 66.3 | 65.3 | 67.4 | −1, 1 death during hr. 3 |
| G | Insulin 1 U. | 66.3 | 21.3 | 18.7 | 24.6 | 36.1 | −32, 2 deaths during hr. 3 & 1 during hr. 4 |
| H | Morphine Sulfate 12 mg. & Insulin 1 U. | 64.9 | 20.4 | 14.8 | 25.3 | 38.2 | −37 |
| I | Morphine Sulfate 25 mg. & Insulin 1 U. | 67.5 | 22.2 | 6.1 | 12.3 | 17.9 | *1, −26, 3 deaths, 1 during hr. 1 & 2 during hr. 2 |
| J | Morphine Sulfate 50 mg. & Insulin 1 U. | 64.6 | 27.9 | 14.2 | 8.1 | 17.6 | *1, −26, 6 deaths, 1 during hr. 2 & 5 during hr. 3 |
| K | Barbital Sodium 100 mg. & Insulin 1 U. | 68.6 | 22.8 | 22.9 | 23.2 | 24.4 | −38 |
| L | Barbital Sodium 200 mg. & Insulin 1 U. | 68.4 | 32.0 | 26.3 | 32.4 | 38.9 | −28. 3 deaths, 2 during hr. 2 & 1 during hr. 3 |

had a hypoglycemia. There were seven deaths in Groups C and D, apparently from respiratory paralysis.[6] Barbital sodium, 100 or 200 mg. slightly depressed the average blood sugar levels except in one case (Table 1, Groups E and F). One unit of insulin, as would be expected, lowered the blood sugar to hypoglycemic levels and even

[6] The blood sugars of these dead animals, in nearly every instance, were decidedly hypoglycemic but they were not included in the average for that hour because the samples had to be obtained from the heart whereas the other samples were obtained from the tail.

caused the death of three animals (Table 1, Group G). The effect of 12 mg. of morphine sulfate (Table 1, Group H) on the hypoglycemic effect of one unit of insulin did not seem significant but there were no deaths in this group. In larger doses (25 and 50 mg.) morphine sulfate (Table 1, Groups I and J) accentuated the hypoglycemic action of insulin except in two instances (both Hour 1). Three of the 10 animals which received insulin and 25 mg. of morphine sulfate died and also 6 of the 10 receiving insulin and 50 mg. of morphine sulfate died. The hypoglycemic action of insulin was not altered significantly by 100 mg. of barbital sodium (Table 1, Group K) but the 200 mg. dose (Table 1, Group L) did have an antagonistic action. Though the hypoglycemic action was not opposed by the 100 mg. dose there were no deaths in this group as compared with three in each of the other two groups (Table 1, Groups G and L).

TABLE 2. EFFECT OF MORPHINE SULFATE ON RECTAL TEMPERATURE AT DIFFERENT ENVIRONMENTAL TEMPERATURES

Average temperatures of four groups of ten rats each, previously fasted for 15–17 hours. Groups A and B kept at room temperature, 20.2°C., throughout and Groups C and D kept in cabinet at 20.3°C. for hours 0 & 1 and thereafter at 29°C. ±1°.

| Group | Hour | | | | | | | Addenda |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | |
| A | 37.1 | 36.8 | No Injection | 36.6 | 36.4 | 36.4 | 36.3 | |
| B | 37.7 | 37.6 | Morphine Sulfate 25 mg. | 35.2 | 34.6 | 37.0 | 37.1 | No deaths |
| C | 37.3 | 37.3 | No Injection | 37.4 | 37.4 | 37.6 | 37.6 | |
| D | 37.6 | 37.4 | Morphine Sulfate 25 mg. | 36.8 | 36.3 | 37.0 | 38.0 | 3 deaths |

Because of the deaths reported in Table 1 when the animals were maintained at 29° C., it seemed advisable to repeat the experiments with morphine at the 25 mg. dose level with a part of the animals maintained at room temperature (20.2° C.) and others at 29° C. When given 25 mg. of morphine sulfate, the group (B) kept at room temperature showed a definite decrease in body temperature whereas the group (D) kept at 29° C. showed only a slight change. It should be noted, however, that in Group D, 3 of the 10 rats died during the four hours following the injection of morphine.

Since the increased environmental temperature augmented the toxicity of morphine, the succeeding experiments summarized in Tables 3 and 4, were performed with only the tail being warmed to facilitate securing of blood samples. The blood sugar values of the corresponding normal Groups A of Tables 1 and 3 are as close as could be expected from two different groups of rats.

The average blood sugar level is not changed significantly (Table 3) by pentobarbital sodium (Groups B and C), evipal sodium (Group

TABLE 3. THE EFFECT OF HYPNOTICS ON BLOOD SUGAR AND RECTAL TEMPERATURE

The average blood sugar results are expressed as mg. per 100 cc. and the average temperature in °C. The number of hyperglycemias observed are designated + and the hypoglycemias −. The bodies of the rats were kept at room temperature but the tails were inserted into a warmed box maintained at 40°C. ±1°. The drugs were injected immediately after the control blood samples were obtained. The number of animals that died before the one hour blood sample was obtained is designated thus *. These animals were replaced to complete a group of ten in each case.

| Group | Experimental Condition | Control | | Hour 1 | | Hour 2 | | Hour 3 | | Hour 4 | | Addenda |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Blood Sugar | Temp. °C. | Blood Sugar | Temp. °C. | Blood Sugar | Temp. °C. | Blood Sugar | Temp. °C. | Blood Sugar | Temp. °C. | |
| A | Normal Group | 69.7 | 37.2 | 66.6 | 37.3 | 66.7 | 37.2 | 64.9 | 37.2 | 65.4 | 37.5 | |
| B | Pentobarbital Sodium 25 mg. | 63.7 | 37.6 | 68.4 | 35.0 | 62.7 | 36.0 | 62.5 | 36.8 | 62.4 | 37.4 | *1 |
| C | Pentobarbital Sodium 35 mg. | 69.2 | 37.2 | 71.7 | 34.4 | 64.5 | 34.9 | 65.8 | 36.2 | 69.3 | 36.8 | *2, 1 death during hr. 2 |
| D | Evipal Sodium 100 mg. | 68.8 | 37.2 | 71.4 | 35.2 | 66.9 | 36.0 | 63.9 | 36.8 | 64.0 | 37.4 | *5, −1 |
| E | Barbital Sodium 100 mg. | 69.9 | 37.1 | 66.4 | 34.6 | 63.7 | 34.0 | 67.2 | 34.6 | 66.7 | 35.2 | |
| F | Barbital Sodium 200 mg. | 68.1 | 37.4 | 70.4 | 35.1 | 65.7 | 34.1 | 66.7 | 33.8 | 72.5 | 33.4 | 4 deaths, 1 during hr. 1, 2 during hr. 2 & 1 during hr. 4 |
| G | Meperidine Hydrochloride 15 mg. | 64.7 | 36.7 | 62.2 | 35.4 | 61.0 | 36.2 | 63.9 | 36.6 | 66.9 | 36.6 | |
| H | Meperidine Hydrochloride 30 mg. | 71.3 | 37.4 | 69.8 | 35.4 | 62.2 | 35.6 | 65.7 | 36.5 | 61.0 | 37.0 | |
| I | Meperidine Hydrochloride 50 mg. | 69.1 | 37.0 | 67.2 | 33.0 | 72.2 | 33.6 | 74.2 | 34.5 | 75.5 | 35.2 | *2, −1, +1 |
| J | Morphine Sulfate 12 mg. | 68.0 | 37.2 | 69.3 | 36.2 | 70.0 | 37.2 | 69.5 | 37.9 | 68.1 | 37.7 | |
| K | Morphine Sulfate 25 mg. | 65.5 | 37.2 | 63.8 | 33.7 | 72.8 | 33.5 | 66.6 | 35.9 | 63.2 | 36.9 | |
| L | Morphine Sulfate 50 mg. | 71.2 | 37.8 | 64.0 | 32.9 | 75.7 | 32.7 | 69.2 | 33.6 | 68.2 | 34.7 | −3, +4 |

to depress the blood sugar level in some instances and several of the rats died, presumably from depression of the respiratory center.[6] Since the peripheral tissues are constantly removing sugar from the blood (Soskin and Levine, 1946a) the glucose content of the tail blood of a moribund rat with a poor circulation from large doses of morphine could be expected to be low. Ingle (1945) in his review has reported that in fasted rats, rapidly induced anoxia causes an initial hyperglycemia which is followed by a hypoglycemia. Van Middlesworth et al. (1944) have found that anoxia in fasted rats causes hypoglycemia. Considering these reports, the depressed respiration (with a concomitant anoxia) due to large doses of morphine sulfate, could be a factor in the hypoglycemias observed in the present experiments. In some individual instances a few of the blood sugars were high enough (Table 1, Groups C and D, Table 3, Groups K and L) to account for the high averages in spite of some very low values. The urine of the rats dying from overdosage was tested for glucose in nearly every instance and found to be negative, evidently the hyperglycemia in these animals had not been high enough to exceed the renal threshold. From their results Feldman et al. (1940) believe that morphine acts on both sympathetic and parasympathetic centers to produce either hyperglycemia or hypoglycemia. This explanation may be applicable to the variations observed in the present investigation.

Barbital was reported by Ellis and Barlow (1924) to produce hypoglycemia whereas Weiss (1926) and La Barre and Vesselovsky (1942) have reported that barbital causes hyperglycemia. In the present study, barbital sodium in the rat (Tables 1 and 3) tended to lower the blood sugar in most instances. With a dose of 200 mg., barbital sodium depressed the rats markedly, causing death in five of twenty animals (Group F, Tables 1 and 3).

Evipal sodium does not alter the blood sugar level of man (Jarman and Abel, 1933) or of the rabbit and guinea pig (Kennedy and Narayana, 1934). In the present study using the rat, evipal also showed no effect on the blood sugar level.

Pentobarbital causes hyperglycemia in dogs (Hemingway et al., 1934) and cats (Smith et al., 1948) but in mice Heistand et al., 1947) and rabbits (Blackberg and Hrubetz, 1936) hypoglycemia is produced. In the present investigation, no significant changes due to pentobarbital were noted in the average blood sugar level in the rat.

Morphine sulfate in small doses (12 mg. Tables 1 and 4) does not seem to have any decided effect on the hypoglycemic effect of insulin. However, there were no deaths in Group H, Table 1, as compared with three deaths in Group G, Table 1, which received only insulin. That morphine in this dosage had an antagonistic action to the hypoglycemic effect of insulin was reported by the author (Larson, 1945) in a preliminary communication after doing most of the experiments reported in Table 1 but not those of Table 4. After repeating some of the

early experiments and having completed those of Table 4, the author feels that his previous conclusion was not completely warranted. At a higher dose level (25 and 50 mg.), morphine sulfate was found in the present study to have an additive effect on the hypoglycemia produced by insulin. This is shown by the low blood sugars, especially at the third and fourth hours as compared with those of the animals receiving insulin only. This retardation in the recovery of the blood sugar level by morphine is comparable to the effects of phenobarbital sodium observed by Johlin (1947) and probably is due to depression of the antagonistic factors (Bouckaret and Duve, 1947) to hypoglycemia. In these four groups receiving 25 and 50 mg. morphine sulfate, ten animals died and two were prostrated. This accentuated toxicity of morphine-insulin combination might have been expected since Seevers and Shideman (1941) reported that morphine decreased the oxygen uptake by the cerebrum. Quastel (1939) has concluded that narcotics produce an anoxemia in those parts of the central nervous system which they affect and this would interfere with oxidation of glucose (already present in a lowered concentration) which is indispensable for the maintenance of the functional integrity of the nervous system (Soskin and Levine, 1946b). That morphine sulfate, causing an anoxia by respiratory depression, could increase the hypoglycemic effect of insulin at hours 2, 3 and 4 might have been expected since Gellhorn and Packer (1940) have reported that short periods of anoxia act antagonistically to insulin whereas longer periods (2 hrs.) aggravated the hypoglycemia. Similar conclusions have been drawn by Van Liere (1942). This depression of respiration by morphine might intensify the respiratory action of insulin since Voegtlin et al. (1924) have shown that insulin in the rat causes pulmonary edema and may cause death by respiratory failure. Lundback (1944) later has reported that insulin lowers the sensitivity of the respiratory center.

Following the injection of barbital sodium (Groups K and L, Table 1, and Groups E and F, Table 4), pentobarbital sodium (Groups E and F, Table 4), pentobarbital sodium (Groups B and C, Table 4) or evipal sodium (Group D, Table 4), the hypoglycemic effect of insulin in nearly every instance was the same or slightly less than during the corresponding periods with insulin alone. It also should be noted that in the group (Group K, Table 1) receiving 100 mg. barbital sodium plus insulin there were no deaths whereas in the group (Group G, Table 1) which received the same dose of insulin without the barbiturate, 3 of the 10 animals died. This protective action of barbital sodium in the rat is similar to the results of Jackson (1931) who found that barbital sodium in rabbits tended to suppress convulsions and deaths due to insulin. Confirmatory evidence of this anticonvulsive action of barbital sodium has been reported (Nakajima, 1938; Andreeva, 1947) and also denied (Okumura, 1939). The depression of metabolism by large doses of barbiturates (Goodman and Gilman,

1941a) would require less glucose for combustion, thereby permitting a higher blood level. This would also be consistent with the view that narcotics in general inhibit oxidation of glucose in the brain (Quastel, 1939) and also the findings of Handley et al. (1941) that pentobarbital reduces the glucose uptake by the brain. In rabbits, Jackson (1931), who used doses varying from 25 to 150 mg. of barbital sodium, found that the 100 mg. dose had the maximum antihypoglycemic effect toward insulin. Since anesthesia with the barbiturates does not alter the insulin content of the pancreas (Haist, 1944), it would seem that any blood sugar effects are extra-pancreatic.

Meperidine hydrochloride, in rabbits receiving a 10 mg. dose, has no effect on the blood sugar level but 20 mg. causes a slight decrease (Barlow, 1947). The action of meperidine hydrochloride in slightly increasing the hypoglycemic action of insulin (Group H, Table 4) might be expected from the action in the normal rat (Group H, Table 3) and also the tendency for the antihypoglycemic action (Group I, Table 4) could be anticipated from the slightly increased blood sugar level (Group I, Table 3). This action of meperidine can be compared to the biphasic action of morphine on the central nervous system (Hazelton and Koppanyi, 1941). The effect of meperidine hydrochloride in producing variations, both hypoglycemia and hyperglycemia in the blood sugar level, was similar to that of morphine sulfate. Since meperidine does not influence the uptake of oxygen by the brain (Elliott et al., 1947), these effects cannot be explained in a manner similar to that proposed previously for the action of morphine. The S-shaped tail reaction, as reported previously by Gruber et al. (1941) for meperidine, was observed.

The normal rectal temperature of the rat has been reported variously from 36.7° C. (Herrington, 1940) to 37.27° C. (Hill, 1947). The temperatures observed in this investigation (Tables 2, 3 and 4) agree with the latter report. The general statement (Goodman and Gilman, 1941b: Sollmann, 1948a) that morphine decreases the body temperature is true of most reports except in the cat (Stewart and Rogoff, 1922b) and the rat (Herrmann, 1941) in which hyperthermia has been reported. In the present investigation from the average temperatures of 40 animals, there was a fall in body temperature produced by the 25 or 50 mg. dose of morphine sulfate (Tables 2 and 3). Morphine sulfate showed a greater toxicity due to the higher environmental temperature (deaths in Tables 1 and 2 compared with Table 3). Chen et al. (1943) have reported an increase in toxicity for certain other drugs when the environmental temperature was increased.

Barbital sodium, pentobarbital sodium and evipal sodium have been reported by previous investigators (Kennedy and Narayana, 1934; Sollmann, 1948b) to lower the body temperature and this effect was observed in the present experiments (Table 3). Meperidine hydro-

chloride (Table 3) also lowered the body temperature of the rat, presumably by central depression.

The body temperature of mice was decreased by insulin (Dudley et al., 1923) and this effect in the rat was observed to a slight degree (Table 4) in this investigation though Visscher and Green (1925) have reported no uniformity of change in rabbits. As previously reported (Voegtlin and Dunn, 1923: Chen et al., 1943; Johlin, 1944), insulin in the present experiments caused a greater hypoglycemic effect in the warmer environment (Group G, Table 1; Group A, Table 4). If Fuhrman's (1946) hypothesis that increasing the temperature increases the enzymatic inactivation of drugs, it would seem that the inactivation of insulin by the tissues is not enzymatic in nature but that the action of insulin is enzymatic as suggested by Soskin and Levine (1946c).

No adequate explanation can be offered for an effect greater than summation on temperature depression of evipal sodium, meperidine hydrochloride or morphine sulfate in combination with insulin nor for an effect less than summation when insulin was combined with pentobarbital sodium or barbital sodium.

## CONCLUSIONS

Morphine sulfate in small doses (12 mg. per kg.) has no effect on the blood sugar level but larger doses (25 or 50 mg.) may produce either a definite hypoglycemia or hyperglycemia.

Morphine sulfate in small doses (12 mg. per kg.) does not affect the hypoglycemic action of insulin but larger does (25 or 50 mg.) prolong the hypoglycemic action.

Barbital sodium (100 to 200 mg. per kg.), pentobarbital sodium (25 mg. or 35 mg. per kg.) or evipal sodium (100 mg. per kg.) do not affect the blood sugar level of the normal animal but slightly decrease the hypoglycemic effect of insulin.

Meperidine hydrochloride (demerol hydrochloride), 15 mg. per kg. had no effect on either the blood sugar level of the normal animal or of the animal receiving insulin. Meperidine hydrochloride 30 mg. per kg., slightly depressed the blood sugar level of the normal rat and also of the animals receiving insulin. Meperidine hydrochloride, 50 mg. per kg., increased the blood sugar level of both the normal animals and of those receiving insulin.

All the drugs used: barbital sodium, pentobarbital sodium, evipal sodium, morphine sulfate, meperidine hydrochloride or insulin, depressed the rectal temperature.

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# THE EFFECT OF ADRENALECTOMY ON THE RENAL TUBULAR REABSORPTION OF WATER IN THE RAT[1]

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It is well known that the adrenal insufficient animal excretes a given dose of water at a slower rate than the normal (Gaunt, 1946). The purpose of the present paper is to present data which indicate that the basis for this deficiency in water excretion is an increased reabsorption of water by the renal tubules in the absence of the adrenal cortical hormones.

## MATERIALS AND METHODS

Three groups of Sprague-Dawley female rats were used; 12 ad libitum fed controls, 11 adrenalectomized and 6 pair-fed controls. The rats weighed from 200-250 grams and were fed GLF "calf meal." The pair-fed controls were started a day following the operated animals, and received only as much ration as the latter had eaten in the previous 24 hr. period. Thus any effects of post-operative inanition were eliminated in interpreting the results. Following adrenalectomy the operated rats were given 1% sodium chloride to drink and were not used for at least two weeks, by which time they had completely recovered from the operation. Daily records of body weight were kept on all animals. The completeness of adrenalectomy was established by the death of all animals within one week after withdrawal of salt.

The creatinine clearance was used as a measure of the rate of glomerular filtration, the technique being modified from Friedman, Polley and Friedman (1947) and Stevenson (1947). A single subcutaneous injection of 5 per cent creatinine was given in amounts of 0.5 ml. per 100 grams of rat. It was found that with this procedure the plasma creatinine at the end of 30 minutes was about 20 mgm. % and remained constant at that level over the following 30 minutes. This fact justified the use of a terminal blood sample in the determination of the creatinine clearance and eliminated the depressive effects of tail cutting as pointed out by Lippman (1948). Accordingly after a 12 hr. fast the rats were given creatinine by subcutaneous injection and 5 ml. water by stomach tube. Thirty minutes later the bladder was emptied manually by suprapubic pressure and a 30 minute collection period begun. Urine collection was made with the rats in large funnels. At the end of the period the bladder was emptied into the funnel, the sides of which were washed with distilled water and the washings added to the original urine. Plasma and

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urine samples were then analyzed for creatinine content. The creatinine clearances obtained by this method agree more closely with recently published values (Lippman, 1948; Corcoran, Masson, Renting and Page, 1948) than with previous observations (Friedman and Livingston, 1942; Dicker and Heller, 1945) which were lower.

## RESULTS

In Table 1 are presented the mean values for the several observations made on each group of animals. In order for two groups of observations to be significantly different, the difference between their means had to be greater than three times the standard error of the difference. By this criterion the rates of glomerular filtration were not different in the three groups, but the urine flow of the adrenalectomized group was significantly lower than that of either control group.

TABLE 1. SUMMARY OF EXPERIMENTS ON THE RATE OF GLOMERULAR FILTRATION AND URINE FLOW IN NORMAL AND ADRENALECTOMIZED, SALT-MAINTAINED RATS

| Groups | Number of animals | Body Weight | Glomerular filtration rate | Urine Flow |
|---|---|---|---|---|
| | | Grams | ml./100cm.²/min. | ml./100cm.²/min. |
| Ad libitum fed controls | 12 | 250±3.30 | .513±.0300 | .0111 ±.00416 |
| Adrenalectomized | 11 | 251±4.80 | .490±.0340 | .00620±.000734 |
| Pair fed controls | 6 | 245±4.80 | .467±.0180 | .0135 ±.00116 |

Figures are the mean of the observations in each group with the standard error. Surface area determined according to the Meeh formula; $SA = 11.23 \times weight^{2/3}$

## DISCUSSION

These data indicate that the adrenalectomized rat maintained on salt has a normal rate of glomerular filtration, and a decreased rate of water excretion which results from an increased rate of tubular reabsorption of water. Birnie, Jenkins, Eversole and Gaunt, (1948) have observed that the blood of adrenalectomized rats contains larger quantities of antidiuretic substance than that of normals. Since it is known that antidiuretic hormone acts to accelerate the tubular reabsorption of water, their observation might constitute an adequate explanation for the increased rate of tubular reabsorption of water reported here. In their paper these authors present a discussion of mechanisms involved.

## SUMMARY

Experiments on rats have shown that the decreased capacity of the adrenal insufficient animal to excrete water is not necessarily due to a decreased rate of glomerular filtration but is rather the result of an increased rate of renal tubular reabsorption of water.

ACKNOWLEDGMENTS

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# HYDROLYSIS OF CONJUGATED SULFATES OF ESTROGENS BY COMMERCIAL ENZYME PREPARATION OF ASPERGILLUS ORYZAE

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MOST of the steroids in freshly voided urine occur as water-soluble esters of either sulfuric or glucuronic acid. In the past, determination of estrogens in urine has been based upon acid hydrolysis of the conjugated estrogens with subsequent extraction of the free estrogens by organic solvents. Van Bruggen, (1948) has recently re-evaluated various methods of acid hydrolysis and estimated the extent of destruction of estrogens during the hydrolysis. Hydrolysis of the conjugated estrogens in human urine by B. coli has been reported by Patterson (1937) to be 75 per cent as efficient as acid hydrolysis.

It has long been known that preparations made from aspergillus oryzae contained a sulfatase. Neuberg et al. (1923a, 1923b, 1926) and Noguchi (1923) described the sulfatase activity of such extracts and claimed that only aromatic ethereal sulfates were subject to hydrolysis by this enzyme. Dzialoszynski (1947) used such an enzyme extract, Clarase, to determine the ethereal sulfate content of equine urine and concluded that this enzyme was inhibited by the urine substrate. We have found, however, complete hydrolysis of the conjugated estrogens in mares' urine using two different commercial enzyme preparations.

Butenandt and Hofstetter (1939) employed Takadiastase as a source of phenolsulfatase for hydrolysis of their synthetic estrone sulfate in order to prove its ester linkage, but no one has reported successful enzyme hydrolysis of urinary conjugated estrogens. We have studied the hydrolysis, by phenolsulfatase preparations, of the conjugated estrogens in pregnant mares' urine and in semi-purified extracts of the conjugated sulfates from such urine. Higher yields of estrogens were obtained by enzyme hydrolysis than by acid hydrolysis. Since enzyme hydrolysis is more specific than acid hydrolysis, much purer extracts are obtained.

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## MATERIALS AND METHODS

Mylase and Mylase P,[1] water-soluble enzyme preparations obtained from aspergillus oryzae, were used as a source of phenolsulfatase.

The urine substrate was prepared by dissolving dried urine[2] of pregnant mares in water. A stable source of comparable urine was thus always available.

The crude conjugated sulfate preparation used was prepared from the above powdered urine as follows: Four extractions were made using 80–90% acetone (2 cc./gm. of urine powder). A two phase separation occurs which makes decantation easy. The acetone extracts were evaporated to dryness in vacuo and the residue suspended in a minimal volume of water. The aqueous suspension was adjusted to pH 3.0 and shaken twice with one-half volume of ether. Ether clears the solution and removes free estrogens, organic acids and other soluble compounds. The ether-washed aqueous solution was adjusted at once to pH 7.0 and extracted four times with one-half volumes of normal butanol. The butanol was removed in vacuo using a Megavac pump and a bath temperature of 50–70°C. The residue from the evaporated butanol extracts was dissolved in a small volume of water and lyophilized. The dried powder (Preparation M) was found to contain 27.7% sulfate, 9.23% sulphur, 4.38% ash and 1.3% of estrogen expressed as estrone. This preparation, like others similarly prepared, gives no precipitate with barium chloride until after slight heating in a solution more acid than pH 4.0.

All enzyme digestions were carried out at pH 6.0 to 6.4 and at a temperature of 50°C., conditions described by Abbott (1947) as optimum for phenolsulfatase. Digestions were carried out in individual tubes (usually 15 ml. centrifuge tubes) for each time interval in the time studies. At the end of the incubation period the digest was shaken with toluene to extract the free estrogens. The tubes were centrifuged and the toluene phase was removed and washed with 10 per cent sodium carbonate. Only one extraction with toluene was made. Fluorimetric determinations were made directly on the toluene phase.

The conjugates in urine powder were acid hydrolyzed by adjusting an aqueous solution of the powder to about pH 1.0 with hydrochloric acid, covering it with a volume of toluene equal to that of the solution and refluxing for 2 to 3 hours. Higher values are obtained when toluene is present during the hydrolysis than when no solvent is used. In the case of Preparation M, hydrolysis at pH 1.0 for 45 minutes was found to yield maximum estrogen values.

The estrogen content of the toluene extracts was determined by the fluorimetric method of Bates and Cohen (1947) using a 4200 Å interference filter as a lamp filter and a 5200 Å interference filter plus a 3387 Corning glass filter as photocell filters. All values were determined using estrone as a standard and are expressed as micrograms of estrone.

## EXPERIMENTAL

In order to determine the quantity of Mylase P required for com-

[1] Wallerstein Laboratories, New York City. In preliminary tests we used successfully a diastatic preparation from aspergillus oryzae obtained from Paul Lewis Laboratories, Milwaukee, Wisconsin.

[2] From Dr. A. D. Odell, Steroid Laboratories Ltd., Montreal, Canada.

plete hydrolysis of the conjugated estrogens, studies were made using the conjugated sulfate Preparation M as the substrate and varying the concentration of enzyme and the time of digestion. The results are presented in Figure 1. It is evident that with a 2 per cent enzyme concentration and after 8 hours digestion, the maximum amount of free estrogen obtained slightly exceeded that obtained with acid hydrolysis. During digestion there was apparently some destruction of estrogens as a result of which the maximum amount of free estrogen,

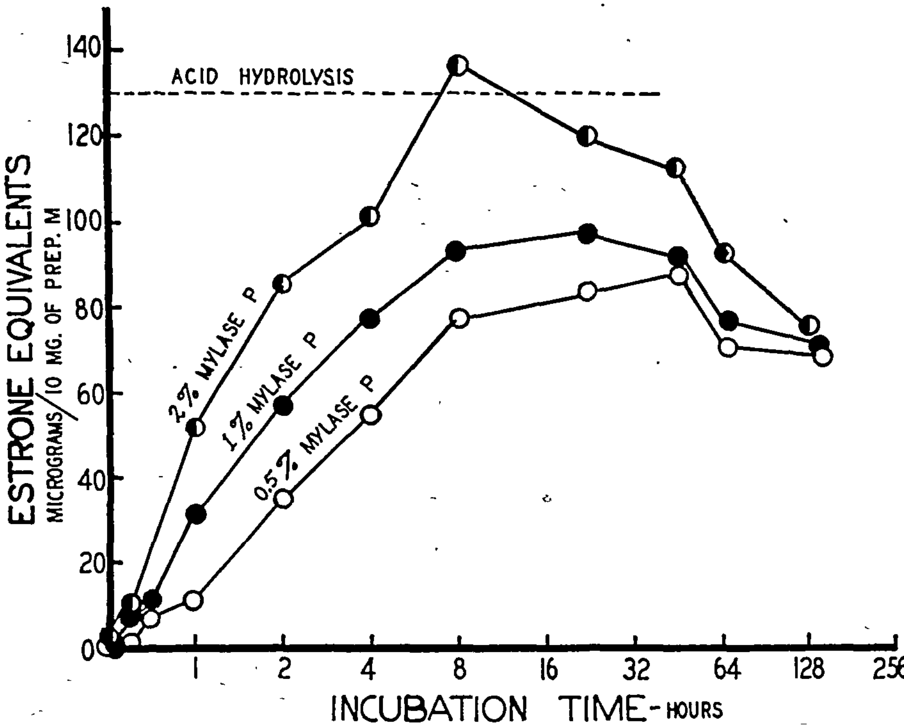


FIG. 1. Rate of enzyme hydrolysis of conjugated estrogen as a function of enzyme concentration at pH 6.0 and at 50°C. Ordinate indicates the amount of free toluene-soluble estrogen. 100 mg. of Preparation M. was dissolved in 5 ml. of water with 100, 50 or 25 mg. of Mylase P respectively in each tube. Controls containing boiled enzyme showed no hydrolysis even after 144 hours.

obtained, with the lower concentrations of enzyme, was only about 70 per cent of that with acid hydrolysis. Controls, consisting of substrate with no enzyme and of substrate plus heat-inactivated enzyme, show no hydrolysis even after 144 hours.

It may be pointed out here that, in order to get as much hydrolysis in 24 hours with enzyme as in 1 hour with acid, the weight of Mylase P used has to be equal to that of Preparation M which contained only 1.3 per cent estrogen. Hence, the phenolsulfatase content of Mylase P is probably very small.

Because the destruction of estrogenic material by Mylase P might

be due to oxidative changes, the series of tests shown in Figure 2 were done. A thick layer of toluene was placed on top of the aqueous digest. This served two purposes: one was to exclude air and the other was to remove the free estrogen from destructive action of the enzymes in the aqueous phase. It is obvious, from the data shown in Figure 2, that the toluene layer in no way inhibits the rate of digestion but does inhibit the destructive action. In addition, the total amount of estrogen extracted by the toluene is about 20 per cent greater than that obtained by acid hydrolysis. The hydrolysis was not complete until

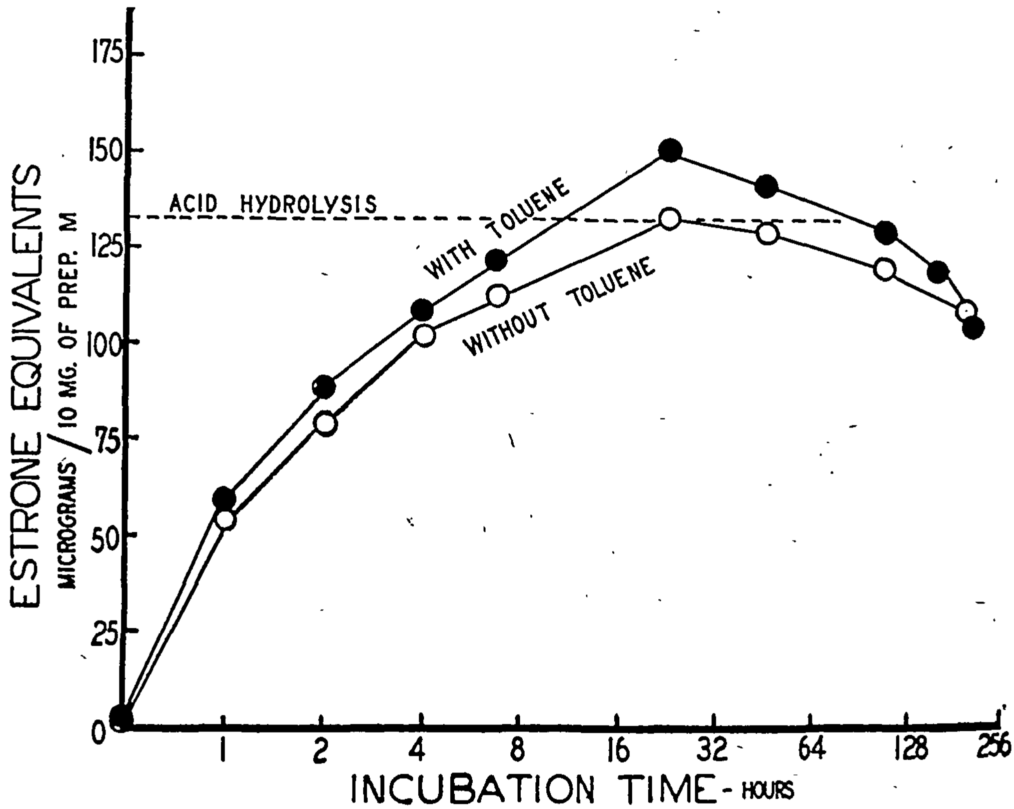


FIG. 2. Rate and extent of hydrolysis of conjugated estrogen with and without toluene at pH 6.0 and 50°C. Ordinate indicates the amount of free toluene-soluble estrogen. 10 mg. of Preparation M and 10 mg. of Mylase P were dissolved in 5 ml. of water in each tube.

24 hours. On longer standing, evidence of destruction was again obtained.

Further indication of the oxidative destruction of the steroid by the enzyme was obtained by subjecting to enzyme digestion for 18 hours two tubes of a solution of pregnant mares' urine powder containing 200 micrograms of estrone equivalent by acid hydrolysis. Both tubes were overlaid with toluene and treated similarly except that air was bubbled through one. The estrogen extracted by toluene from the solution in the tube incubated without air was equivalent to 220 micrograms of estrone. Only 120 micrograms, on the other hand,

were extracted by toluene from the tube which had air passed through it.

In Figure 3 are shown the results obtained upon incubation of a freshly prepared solution of pregnant mares' urine powder with Mylase P, both with and without an overlay of toluene. Once again, the destructive process is evident in the tubes incubated without toluene, while some inhibition of this phenomenon is observed when the tubes are kept covered with toluene. In addition, the total amount of estrogen extracted by the toluene is about 30 per cent greater than that obtained by acid hydrolysis. Controls, consisting of substrate with no

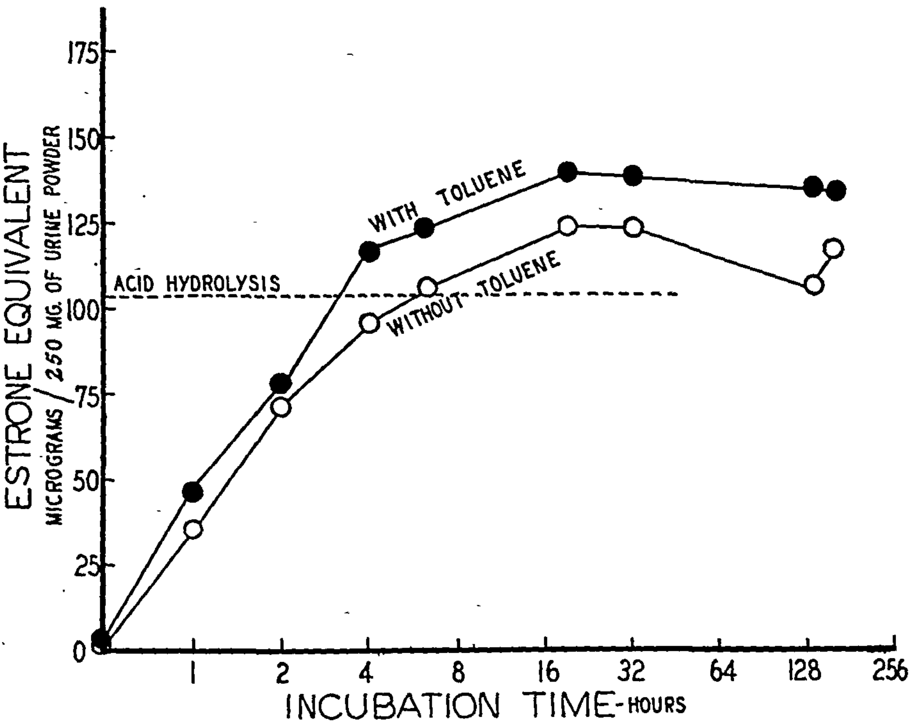


FIG. 3. Rate and extent of hydrolysis of conjugated estrogen with and without toluene at pH 6.4 and 50°C. Ordinate indicates the amount of free toluene-soluble estrogen. 250 mg. of urine powder and 40 mg. of Mylase P were dissolved in 5 ml. of water in each tube.

enzyme and of substrate plus heat-inactivated enzyme, gave no hydrolysis even after 144 hours.

The increased rate of destruction by Mylase P of the estrogens in the conjugate Preparation M, as compared to the rate of destruction in the reconstituted urine, may be due to the removal of protective substances during the purification of Preparation M since 98 per cent of the solids present in the urine were removed during its preparation. Similar observations have been made by Smith and Smith (1941) who found that crystalline estrone in aqueous solution will have 50 per

cent of its potency destroyed by acid hydrolysis at 100°C. for 20 minutes, while in the presence of urine such destruction does not occur.

Having established the effectiveness of Mylase P and the optimum hydrolyzing conditions, larger amounts of substrate were subjected to enzyme hydrolysis and then fractionated. Fractionation was carried out according to the scheme shown in Figure 4.

FIGURE 4

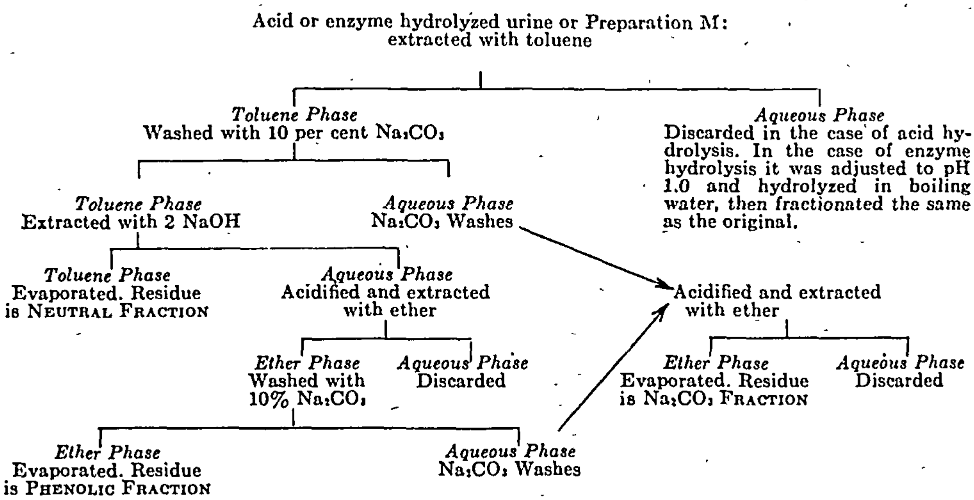


Table 1 is a compilation of the results obtained upon the subjection of 1 gram of conjugated sulfate Preparation M to enzyme and acid hydrolysis in the presence of toluene. The superiority of enzyme hydrolysis over acid hydrolysis is shown; 1) by higher yield of estrogen in the phenolic fraction with lower weight of non-estrogenic material, which means greater purity, 2) by much less color at all stages of fractionation and 3) by the crystalline nature of the phenolic fraction from enzyme hydrolysis.

During acid hydrolysis, many toluene-soluble impurities are formed. This is indicated by the fact that acid hydrolysis of the aqueous residue remaining after toluene extraction of the enzyme digest (Line 3, Table 1) yields fractions which, when added to the fractions obtained after enzyme digestion (Line 2, Table 1) give totals approximately those obtained after only acid hydrolysis of Preparation M (Line 1, Table 1). The phenolic fraction is an exception.

Table 2 presents data obtained in subjecting a freshly prepared solution of pregnant mares' urine powder to enzyme hydrolysis or acid hydrolysis. Again the efficiency of enzyme hydrolysis is very well illustrated by first, a greater yield of estrogens than the yield obtained by acid hydrolysis, and second, by the smaller amount of impurities obtained. In the phenolic fraction, isolated after enzyme hydrolysis, 20 per cent of the weight is estrogenic material while in the

TABLE 1. COMPARISON OF ENZYME AND ACID HYDROLYSIS OF 1 GRAM OF CONJUGATED ESTROGEN PREPARATION M CONTAINING AN ESTIMATED 13 MG. OF ESTROGEN. THE AQUEOUS PHASE REMAINING AFTER ENZYME HYDROLYSIS (LINE 2) WAS ACID HYDROLYZED (LINE 3). A LAYER OF TOLUENE WAS PRESENT DURING ALL DIGESTIONS

| Experimental Procedure | $Na_2CO_3$ Fraction | Neutral Fraction | | | Phenolic Fraction | | |
|---|---|---|---|---|---|---|---|
| | Weight | Weight | Optical Density* | Estrogen Content | Weight | Optical Density* | Estrogen Content |
| | mg. | mg. | | mg. | mg. | | mg. |
| Acid Hydrolysis:<br>1. 1 gm. conjugate M in 50 ml. $H_2O$ at pH 1.3 Refluxed 45 minutes | 32.0 | 18.2 | 1.3 | 0.45 | 137.0 (tar) | 0.73 | 11.9 |
| Enzyme Hydrolysis:<br>2. 1 gm. conjugate M 1 gm. Mylase P 50 ml. $H_2O$ at pH 6.0 20 hrs. at 50° C. | 9.0 | 8.0 | 0.17 | 0.23 | 39.0 (crystals) | 0.47 | 13.0 |
| Acid Hydrolysis:<br>3. Aqueous phase from (2) Adjusted to pH 1.2 Refluxed 50 minutes | 21.2 | 12.0 | 1.5 | 0.10 | 9.5 (tar) | 0.56 | 0.25 |

* Optical density at 420 mu. of a solution of the entire fraction in 25 ml. of ethanol.

TABLE 2. COMPARISON OF ENZYME AND ACID HYDROLYSIS OF ONE KILO OF URINE POWDER EQUIVALENT TO ABOUT 20 LITERS OF PREGNANT MARES' URINE. THE AQUEOUS PHASE REMAINING AFTER ENZYME HYDROLYSIS (LINE 2) WAS ACID HYDROLYZED (LINE 3). A LAYER OF TOLUENE WAS PRESENT DURING ALL DIGESTIONS.

| Experimental Procedure | $Na_2CO_3$ Fraction | | Neutral Fraction | | Phenolic Fraction | |
|---|---|---|---|---|---|---|
| | Weight | Estrogen Content | Weight | Estrogen Content | Weight | Estrogen Content |
| | gm. | mg. | gm. | mg. | gm. | mg. |
| Acid Hydrolysis:<br>1. 1 kilo of urine powder in 10 liters of $H_2O$ at pH 1.0 Reflux 3 hours | 79.0 | 0 | 2.3 | 43.0 | 12.1 | 540.0 |
| Enzyme Hydrolysis:<br>2. 1 kilo of urine powder with 100 gm. Mylase P in 10 liters of $H_2O$ at pH 6.2 18 hours at 50°C. | 1.5 | 0 | 0.36 | 13.3 | 3.25 | 683.0 |
| Acid Hydrolysis:<br>3. Aqueous phase from (2). Adjusted to pH 1.0 Refluxed 3 hours | 73.0 | 0 | 2.80 | 8.75 | 5.5 | 35.0 |

fraction isolated after acid hydrolysis, only 5 per cent of the weight is estrogenic material. The weights of the carbonate-soluble and neutral fractions show a still greater difference between enzyme and acid hydrolysis.

Preliminary experiments with human pregnancy urine (5th month gestation) indicate that a portion of the estrogens, presumably sulfates, is hydrolyzed by Mylase P.

## CONCLUSIONS

From the data in Table 1 and 2 it may be concluded that the hydrolyzing activity of the enzyme is largely due to a phenolsulfatase because the weight of the neutral fraction obtained after enzyme hydrolysis relative to that obtained after acid hydrolysis, is only about 44 per cent and 16 per cent respectively. This indicates that part of the conjugated sulfates, presumably non-phenolic conjugates, is not hydrolyzed by the enzyme. Hence, Mylase P does not contain a general sulfatase.

The phenolsulfatase specificity of Mylase P is further indicated by the fact that Mylase P does not hydrolyze phenolphthalein glucuronide. Hence, it does not contain a general phenolesterase (Fishman, 1948).

Our data support the conclusions of Noguchi (1923) that aspergillus oryzae extracts contain a specific phenolsulfatase.

## SUMMARY

Mylase P, a commercial enzyme preparation from aspergillus oryzae, was found to hydrolyze the conjugated estrogens present in the urine of pregnant mares. After 24 hours digestion at pH 6.0 and 50°C. under a layer of toluene with relatively large amounts of Mylase P, about 20 per cent higher yields of estrogens were obtained than after acid hydrolysis.

The phenolic fraction isolated after enzyme hydrolysis contained a much higher percentage of estrogens than that isolated after acid hydrolysis.

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# PRELIMINARY OBSERVATIONS ON THE ANTIDIURETIC SUBSTANCE RECOVERED FROM THE URINES OF PATIENTS WITH CIRRHOSIS OF THE LIVER

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In 1945 Ralli et al. reported an increase in the amount of antidiuretic substance present in the urines of patients with cirrhosis of the liver and ascites. Recently Drill and Frame (1948) have confirmed this observation. The presence of increased amounts of an antidiuretic substance in the urine of patients with water retention is of interest because of the possible relationship that the substance in urine bears to the antidiuretic hormone of the posterior pituitary and because of the rôle of this hormone in urine flow (Wesson, 1948) and therefore in water balance (Gilman and Goodman, 1936). Verney (1946, 1947) and Pickford (1945) have shown that the antidiuretic hormone regulates the rate of urine flow, and Verney (1947, 1948) has described the mechanism for the control of the secretion of the hormone. Although much is known of the properties of the hormones of the posterior pituitary gland and the mechanism of their elaboration, many gaps in our knowledge of these hormones remain, and this is especially true of the antidiuretic hormone (Chow, 1944; Van Dyke, 1943).

In an effort to study the role of the antidiuretic substance in the formation of ascites in patients with cirrhosis of the liver, it was thought advisable to study the nature of the antidiuretic substance in urine. This report deals with the initial phases of the processing of the urines from patients with cirrhosis of the liver.

## PROCEDURE AND RESULTS

Urines from patients with cirrhosis of the liver in various stages of the disease were collected and pooled. Toluene and sufficient 25% acetic acid to bring the urine to pH 4 were used as preservatives. The urine was processed in batches of from 1 to 5 liters.

*Dialysis.* Each liter of urine was divided into 200 cc. aliquots, and each aliquot was contained in a membrane made of Visking cellulose

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sausage casing (36/32 Nojax). Five of these aliquots were dialyzed against 5 gallons of distilled water in an earthenware crock for 5 days. The dialyzing water was changed once or twice a day for the first 3 days and daily for the last 2 days. Both the urine and the water were protected against decomposition with toluene. It is probable, in the light of subsequent experiments, that a considerable loss of the antidiuretic substance occurred during this prolonged dialysis. This point will be discussed in more detail later.

*Concentration of the Dialysate.* After dialysis each aliquot, in the same casing in which it had been dialyzed, was concentrated by suspension in front of a fan. This required approximately 24 hours.

In order to establish the degree of concentration necessary to obtain an antidiuretic effect after prolonged dialysis, samples from individual patients were concentrated to different extents ranging from 1/8th to 1/68th of the original volume. They were then tested for antidiuretic activity by injection into hydrated rats, as previously described (Ralli et al., 1945). The results, which are shown in Table 1, indicate that urine which has been dialyzed for long periods must be concentrated at least to 1/40th of its original volume before a positive antidiuretic test can be obtained. Samples "C" and "E" were tested at 2 different levels of concentration, and only when the concentration was about 40 times that of the original urine was a positive antidiuretic test obtained. In the subsequent processing, dialyzed pooled urines were therefore concentrated to 1/40th of their original volumes.

TABLE 1. RELATIONSHIP OF THE DEGREE OF CONCENTRATION OF DIALYZED URINE TO THE BIOLOGICAL TEST FOR THE ANTIDIURETIC SUBSTANCE

| Urine Sample | Initial Volum ml. | Concentrated Volume ml. | Concentration Factor | Biological Test |
|---|---|---|---|---|
| H | 2,820 | 350 | 8 | Negative |
| J | 700 | 42 | 17 | Negative |
| A | 4,475 | 208 | 22 | Positive |
| F | 1,900 | 73 | 26 | Negative |
| D | 7,180 | 200 | 36 | Negative |
| G | 4,100 | 108 | 38 | Negative |
| I | 966 | 25 | 39 | Positive |
| M | 2,300 | 55 | 42 | Positive |
| L | 1,700 | 35 | 49 | Positive |
| B | 5,925 | 100 | 59 | Positive |
| K | 4,850 | 80 | 61 | Positive |
| C | 5,440 | 300 | 18 | Negative |
| C | 5,440 | 80 | 68 | Positive |
| E | 1,820 | 175 | 10 | Negative |
| E | 2,000 | 50 | 40 | Positive |

The concentrated urine was then filtered in the cold, using a coarse filter paper. The filtrate was tested biologically for its antidiuretic activity and was found to contain significant amounts of antidiuretic substance.

*Chromatographic Treatment.* The filtrate was then chromatographed, using a column of Permutit prepared according to Folin. Potts and Gallagher (1944) have shown that the pressor principle of Pitressin can be separated from the oxytocic principles by chromatographic procedures using Permutit. We did no analyses for the pressor or oxytocic principles, but rather have studied the behavior of the antidiuretic substance on the Permutit column and the eluation of this substance by 5% NaCl-1 M acetic acid.

The columns were 42.5 cm. long (with an inside diameter of 1.5 cm.) and contained 35 gm. of Permutit. The bottom of the tube contained a glass wool plug and a rubber stopper through which a small piece of capillary tubing was inserted. The Permutit was packed into the column and then washed with 300 cc. of distilled water, using suction. The material to be chromatographed was delivered to the column from a separatory funnel suspended above it, at such a rate that the upper surface of the column had a continuous flow of material presented to it. It was found in initial experiments that 35 gm. of Permutit was not sufficient to adsorb all the antidiuretic substance in 35 ml. of concentrated urine, but that 2 gm. of Permutit was necessary for each ml. of concentrated urine.

The urine was allowed to flow down the column by gravity; and when the last of the concentrate had left the upper surface of the column, distilled water was allowed to flow through the column and the eluates were collected. When some 5 to 6 eluates had been collected, 5% NaCl in 1 M acetic acid was passed through the column in a manner similar to that described for the water. Again eluates were collected, the point of separation being determined by the change in the pH of the effluent, as the NaCl-acetic acid solution passed through the column. Each eluate was tested biologically for its antidiuretic properties by injection into rats.

If sufficient Permutit was used, negligible quantities of the antidiuretic substance were found in the water eluates. When the column was eluted with 5% NaCl in 1 M acetic acid, the pH of the eluates decreased from 6.8 to 3.4. No significant antidiurectic effect was found until the pH approached 4. At this pH a high level of antidiuretic activity was demonstrable. The activity of this eluate is shown graphically in Figure 1, in which the effect of the injection of 1 ml. of the eluate on the urine output of hydrated rats (solid circles) is compared to the effect of the injection of 1 ml. of distilled water (clear circles).

In the original experiment the antidiuretic activity was confined to a single eluate. In later experiments, in which the original urine concentrates had a higher antidiuretic activity, several of the eluates were found to have a pronounced antidiuretic effect. The antidiuretic activity in the later experiments was present in eluates in which the pH ranged from 5.1–3.9.

The concentrated material prepared by dialysis, insipation, and elution from a Permutit column retained its antidiuretic activity for 4 months when stood in the icebox under toluene. On standing a precipitate formed which had no antidiuretic effect. The supernatant containing the antidiuretic material was clear and dark brown in color.

*Relationship of Dosage to Response of the Antidiuretic Substance.* The methods of assay for the antidiuretic effects of extracts of urine,

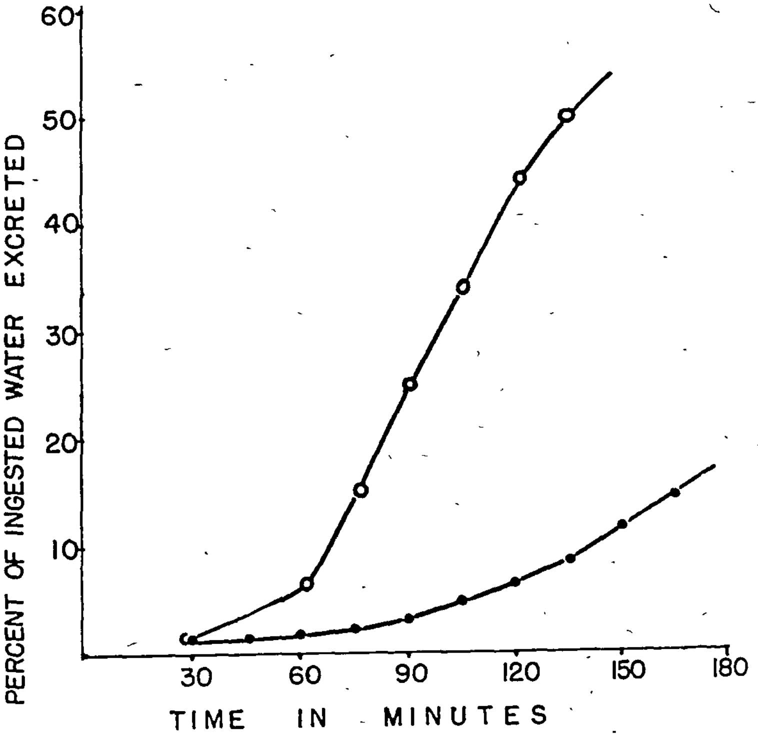


FIG. 1. The antidiuretic effect of a NaCl—$CH_3COOH$ eluate when injected into hydrated rats as compared with the effect of 1 cc. of distilled water.

blood and tissues have depended on the suppression of urine secretion when such extracts were injected into experimental animals or man (Robinson and Farr, 1940; Teel and Reid, 1939; Ralli et al., 1945; Burn, 1931; Drill and Frame, 1948).

Jeffers et al. (1942) have shown that the response to a given dose of pituitrin is logarithmically related to the dose. The data published by Ralli et al. (1945) has been recalculated and the same relationship was found to hold true. Because of the importance of this relationship in the interpretation of the bioassays, additional experiments were carried out, the results of which are given in Figure 2. Rats that had been fasted and deprived of water for 17 hours were hydrated to 5% of their body weight by stomach tube and injected intraperitoneally with varying dilutions of commercial Pitressin. Urine was collected at 15-minute intervals for a period of 185 minutes. Over the range of

dosage studied, 2.5 to 10 milliunits per milliliter, the logarithmic relationship was found to hold, thus confirming the orginal observations of Jeffers (1942).

*Effect of the Tonicity of the Solution Containing the Antidiuretic Substance upon the Response.* Verney's experiments (1947) have shown that the intravenous injection of hypertonic solutions of NaCl will cause a decrease in urine flow which he attributes to an increased secretion of the antidiuretic hormone. This effect was not evoked after removal of the posterior pituitary.

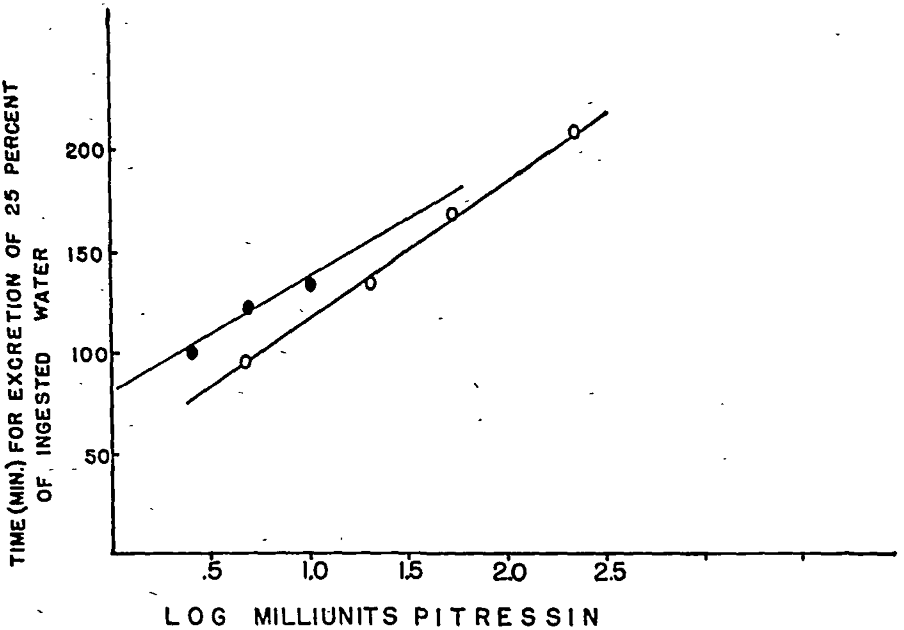


FIG. 2. Relationship of log. dosage of Pitressin to time of excretion of 25% of water ingested

(Solid circles) .................................. Present Authors

(Open circles) ................ Data recalculated from Ralli et al.

We have tested the effects of the intraperitoneal injections of 1 cc. of NaCl solutions varying in concentration from 2.5 to 10%. The results are shown in Figure 3. The 5% and 10% solutions caused a decrease in urine output. The shape of the curve following the injection of the 5% NaCl solution is not characteristic of the response elicited in rats by the injection of Pitressin, although there is evidence of some antidiuretic effect. The response to the injection of 10% NaCl approximates that of Pitressin although it is not identical to it. The animals which had been given 2.5% NaCl intraperitoneally, a concentration roughly equivalent to that of the NaCl eluates from the Permutit column, excreted 25% of the injested water within a normal time.

*Effect of Dialysis on Antidiuretic Activity.* A solution containing the

antidiuretic substance as eluted from a Permutit column with NaCl was adjusted to a pH of 3.5 with acetic acid. (The antidiuretic hormone has been reported to be most stable at pH 3 by Van Dyke, 1943.) The solution of the antidiuretic substance was then dialyzed for 4 hours against running water, after which the material remaining in the casing was tested for its antidiuretic effect. In Figure 4 the

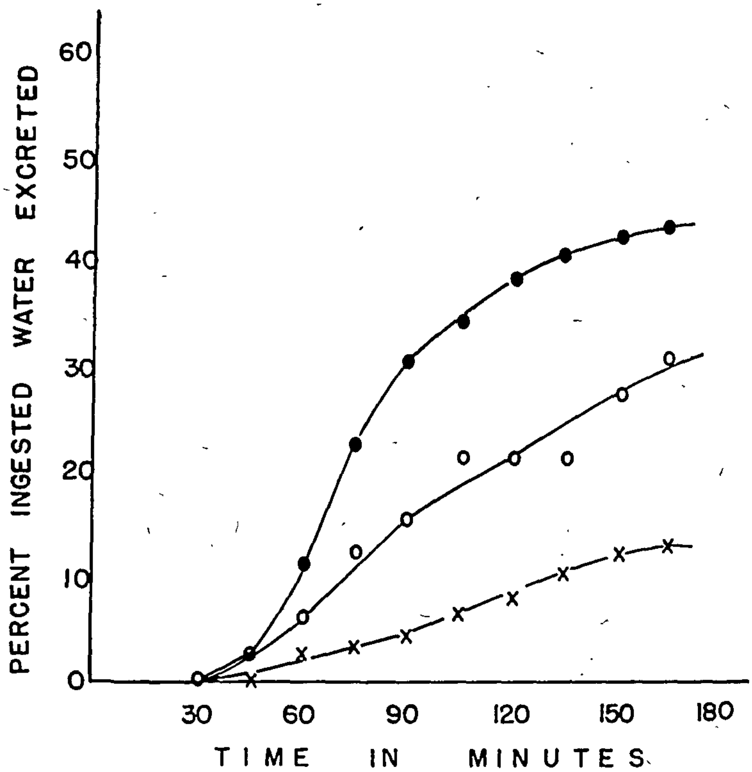


FIG. 3. Showing the effect of injection of 1 cc. of hypertonic sodium chloride intraperitoneally on the urine excretion of hydrated rats.
(Solid circles) .................................... 2.5% NaCl
(Open circles) .................................... 5.0% NaCl
(Crosses) .................................... 10.0% NaCl

response of rats injected with a suitable concentration of the extract before dialysis is compared with that of rats injected with the same dilution of the dialyzed extract. It is apparent that a loss or destruction of antidiuretic activity occurred as a result of dialysis.

Table 3 shows the concentration of antidiuretic substance in 3 solutions before and after dialysis. The loss ranged from 50% in concentrate A, which originally contained 5 units of antidiuretic substance per cc., to 90% in concentrate C, which originally contained 40 units per cc. It is of interest that in these experiments the per cent of the antidiuretic activity lost was directly proportional to the logarithm of the concentration originally present. This relationship cannot be readily interpreted on the assumption that the loss was due to

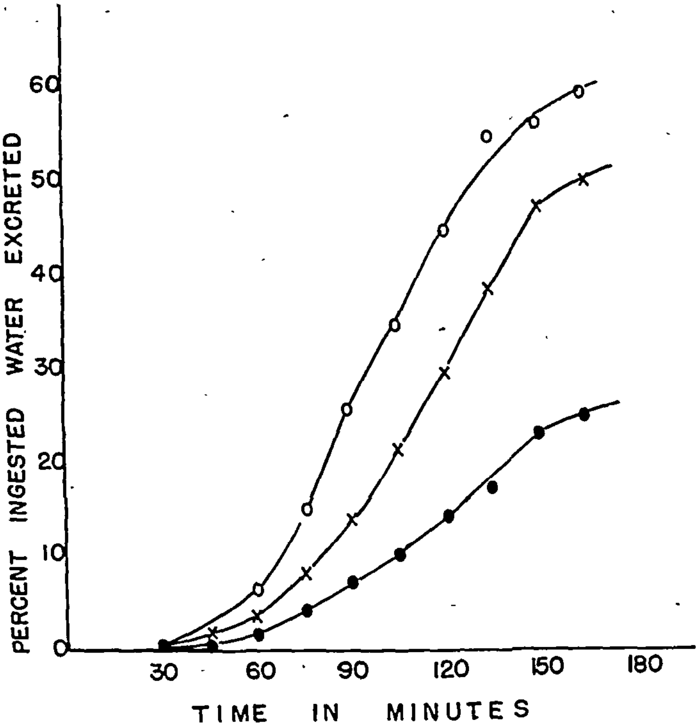


FIG. 4. Showing response of test animals to the antidiuretic substance from cirrhotic urine before and after dialysis
(Open circles) Controls injected with distilled water
(Crosses) A urine concentrate eluted from Permutit and then dialyzed
(Solid circles) The same concentrate as above, undialyzed

diffusion through the dialyzing membrane, and at present we are unable to explain it.

TABLE 3. LOSS OF ANTIDIURETIC ACTIVITY BY DIALYSIS

| Urine Concentrate | Concentration of Antidiuretic Substance (Units/Ml.) | | Per Cent Loss |
|---|---|---|---|
| | Before Dialysis | After Dialysis | |
| A | 5.0 | 2.5 | 50% |
| B | 13.9 | 3.9 | 70% |
| C | 40.0 | 4.0 | 90% |

The concentration of the antidiuretic substance is expressed as the amount equivalent to 1 milliunit of Pitressin per milliliter (Parke-Davis and Company).

## DISCUSSION

From these preliminary studies we are not yet in a position to state the nature of the antidiuretic substance present in urine. The observations point to the fact that such a substance is present in urine, although with the ordinary methods of dialysis considerable portions of the substance will be lost. However, if large volumes of

urine are sufficiently concentrated, a substance can be obtained by the methods described which has a very definite antidiuretic effect.

Recently Mazur and Shorr (1948) have identified ferritin as a vasodepressor material with an antidiuretic effect. It seems unlikely that the antidiuretic activity of the extracts we have studied can be attributed to ferritin. First, except in severe kidney damage very little ferritin is excreted in the urine. Second, the antidiuretic substance which we have concentrated loses its antidiuretic effect in neutral or alkaline solutions, while ferritin is stable and exerts its antidiuretic effect at a pH of 7.4.

## SUMMARY

Urines from patients with cirrhosis of the liver were collected, dyalyzed, and concentrated. The concentrates were adsorbed on a Permutit column and fractionally eluted with distilled water and with 5% NaCl in 1 M acetic acid. An active antidiuretic substance of high potency was obtained in the NaCl eluates. Dialysis reduced the antidiuretic potency of the eluates to less than half their original value. The eluates retained their potency when refrigerated for 4 months.

Although these studies do not define the nature of the antidiuretic substance present in urine, they do establish the fact that such a substance is present, that its antidiuretic effect when injected into rats is similar to that of commercial Pitressin, and that ordinary methods of dialysis may result in the loss of varying amounts of the material.

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# THE EFFECTS OF PITUITARY GROWTH HORMONE ON THE METABOLISM OF ADMINISTERED AMINO ACIDS IN NEPHRECTOMIZED RATS[1]

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THE anterior pituitary growth hormone is known to promote the retention of nitrogen in the body. This effect is best seen when observations are made in animals fed adequate protein over a day or more. Anterior pituitary extracts will lower the plasma amino nitrogen of fasted animals within a few hours, an effect presumably also the result of growth hormone action. From the conditions of these experiments, it is not possible to say whether the effects seen are due to suppression of protein catabolism or to an increase in the rate of protein anabolism. An acute effect of the growth hormone on the retention of administered nitrogen has not heretofore been demonstrated. In the present experiments, the rate of increase of urea in the blood of nephrectomized rats has been used as a measure of the rate of protein metabolism. A mixture of amino acids in the form of a casein hydrolysate was given intravenously, and observations were made of the effects of a partially purified growth hormone preparation on the subsequent urea formation. No change was seen in the basal rate of nitrogen catabolism under the conditions of these experiments, but a marked decrease was observed in the amount of urea formed after the administration of the amino acid mixture. These observations suggest that the action of the growth hormone was here not so much the suppression of protein catabolism as the promotion of protein synthesis from exogenous amino acids.

## METHODS

The procedure used in these experiments was similar to that recently described by Engel, Pentz, and Engel (1948), except that observations on the blood urea were begun a few hours after removal of the kidneys, instead of the next day. The lower blood urea levels at the earlier time make it possible to measure smaller differences accurately and so to observe changes within shorter time intervals than those used by Engel et al.

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The experimental animals were male white rats of the Sprague Dawley strain, about 3 months old and weighing between 300 and 350 gm. The animals had been fed on a diet of Purina Laboratory Chow. All animals were fasted overnight before experiment. The kidneys were removed through a lumbar approach, with the animals under light pentobarbital anesthesia (3 mg. per 100 gm. sodium pentobarbital given intraperitoneally) supplemented with ether during the first stages of the operation. The animals all regained consciousness fully within an hour after operation and were then allowed to rest quietly in a warm, lighted place until the later observations were begun. About 3 to 3½ hours after the removal of the kidneys, the animals were reanesthetized lightly with pentobarbital (2 to 3 mg. per 100 gm.). A blood sample was then taken from the cut tail, and immediately afterwards, the solution to be administered, the amino acid mixture or saline, was injected through the femoral vein near the groin. No further anesthetic was administered, later blood samples being taken from the tail while the animal lay quietly, wrapped in a towel.

The mixture of amino acids given was a sterile solution of material derived from casein by enzymatic hydrolysis.[2] This was dissolved as a 10 per cent solution, partially neutralized to pH 6.6 by the addition of 0.6 ml. of 0.5 N NaOH per gm. of solids. This solution was filtered by suction, dispensed into small serum vials, and immediately sterilized by autoclaving for 20 min. at 15 lb. pressure. Once tapped, a vial of amino acid solution was not used again. The solution used in most of these experiments contained per ml., 11.1 mg. of total nitrogen by Kjeldahl analysis, 7 mg. of amino nitrogen by the $\beta$ naphthoquinone method, and from the manufacturer's estimate 10 mg. of potential amino nitrogen. One ml. of the amino acid solution per 100 gm. body weight was injected slowly over a period of 1 to 2 minutes. If the solution was not injected too rapidly, the animals appeared to suffer no untoward reactions, a brief period of hyperventilation being the only disturbance noted.

In control experiments, freshly prepared 0.9 per cent sodium chloride solution was injected intravenously. One-half ml. was given per 100 gm. body weight. This volume contained about the same amount of inorganic salts as was contained in the 1 ml. of amino acid solution.

Blood samples of 0.2 to 0.4 ml. volume were taken from the tail before the administration of the amino acids or salt solution and at intervals afterwards for the estimation of urea, amino nitrogen, glucose, and lactate. The total volume of blood withdrawn was from 0.8 to 1.8 ml. or not more than 0.6 ml. per 100 gm. body weight. Whole blood was used for all analyses unless otherwise indicated. In most of the nephrectomized animals, estimations of all the constituents listed were made on the initial samples; urea nitrogen was then determined at 1, 3, and 20 hours after the injections, amino nitrogen at 15, 30, and 60 minutes, glucose at 1 and 3 hours, and lactate at 30 min., 1 hr., and 3 hours. In some experiments observations of blood urea only were made on samples taken initially and at 1 and 3 hours; no differences were seen in the rates of urea formation in animals so treated from those bled more extensively. When plasma amino nitrogen was determined, samples were taken initially and at 10, 30, and 60 minutes after the injections; other determinations were not made in this series of experiments.

[2] Amigen, prepared by Mead Johnson and Company, Evansville, Indiana. We wish to thank Dr. Warren M. Cox of that company for generously supplying us with this preparation.

The blood samples were deproteinized with tungstic acid or with copper tungstate. The plasma samples were prepared as follows: About 0.4 ml. of blood was drawn into a 1 ml. centrifuge tube containing a small amount of potassium oxalate, stirred well with a fine glass rod, and spun 5 minutes in an angle-head centrifuge. Two-tenths ml. of plasma was then withdrawn with a Levi pipette, delivered into 5 ml. of 0.01 N sulfuric acid, and mixed. To the diluted plasma 0.2 ml. of 3.0 per cent sodium tungstate was then added and mixed thoroughly. The mixture was allowed to stand until settling had begun and then centrifuged. This procedure was shown to allow quantitative recovery of added amino acids estimated by the $\beta$ naphthoquinone method as used here. It has been found essential for this recovery to use less tungstate in proportion to the amount of plasma than is customarily used for whole blood, since traces of tungstate not precipitated with the proteins inhibit color development during the analysis.

Chemical analyses were made by the following methods: For urea, the xanthydrol method as described by Engel and Engel (1947) was applied to 1 ml. aliquots of the supernatants prepared from whole blood by tungstic acid precipitation, or the method of Archibald (1944) was applied to samples deproteinized with copper tungstate. For amino nitrogen, the photometric procedure of Frame, Russell, and Wilhelmi as modified by Russell (1943, 1944) was used with 1 ml. of the supernatant from whole blood (1:28 dilution) or with 2 ml. of that from plasma. For glucose, the Nelson arsenomolybdate reagent was used in conjunction with Somogyi's phosphate-buffered copper reagent on 0.5 ml. aliquots, and for lactate, the Barker-Summerson method was applied to 0.3 ml. samples of the supernatants from whole blood. All determinations were made in duplicate, or in the case of urea, in some cases in triplicate.

The anterior pituitary growth hormone preparation used in these experiments was a partially purified fraction prepared by the addition of alcohol to an alkaline extract of beef anterior lobes, the "C" fraction described by Fishman, Wilhelmi, and Russell (1947). This fraction is precipitated at pH 6.8 and 24 per cent ethyl alcohol. From electrophoretic analysis, it appeared to contain approximately 40 to 50 per cent growth hormone and smaller amounts of some other components. It was highly potent in promoting growth, inducing an average gain of 21 gm. in 10 days when given at a dose of 100 $\mu$g. per day to hypophysectomized rats weighing about 100 gm. The weights of the thymuses, expressed per 100 gm. body weight, increased 50 per cent in the 10 day test, as compared to the weights in untreated controls. When given either at the 100 $\mu$g. dose for 10 days or at a dose of 1 mg. per day for 4 days, the preparation induced no changes in the weight or appearance of the adrenals, thyroids, testes, or accessory sex organs. There was therefore probably little gonadotrophin present, but there may have been some thyrotrophic hormone; from other evidence, there probably was also some lactogenic hormone. The preparation was also tested for the presence of adrenocorticotrophic hormone by the sensitive assay method of Sayers, Sayers, and Woodbury (1948). Fifty micrograms given intravenously per 100 gm. body weight to hypophysectomized rats produced an average fall in adrenal ascorbic acid of $9 \pm 4$ mg. per cent. Since a significant response may be induced by 0.1 to 0.2 $\mu$g. of highly purified adrenocorticotrophin, the growth hormone preparation used here appeared to be practically free of this factor.

The hormone preparation was stored in the cold in the solid lyophilized state; for use, it was suspended in 0.9 per cent sodium chloride and dissolved by adjustment of the pH to 8 to 9. This solution was prepared freshly on the day of use. A volume of 0.3 ml., containing 1 mg. of material per 100 gm. body weight, was injected intraperitoneally 1½ to 2 hours before experiment (1½ to 2 hours after removal of the kidneys).

## OBSERVATIONS

*Urea formation in untreated animals:* A summary of the data obtained on the basal rates of urea formation in nephrectomized rats and comparison of these rates with those obtained by Engel, Pentz and Engel in similar experiments are presented in Table 1. In confirmation of the latter authors, the basal rate of urea formation was found quite constant over periods up to 22 hours after nephrectomy. This rate was about ⅓ lower than that reported by Engel, et al. The reason

TABLE 1. BASAL RATES OF UREA FORMATION IN NEPHRECTOMIZED RATS

| | Length of observation period | Time after operation | No. of observations | Increase in blood urea nitrogen | |
|---|---|---|---|---|---|
| | hrs. | hrs. | | mg. per cent per hour | standard deviation |
| I. Combined series, Tables 2 and 6* | | | | | |
| A. | 1 | 3–4 | 23 | 3.38 | 1.09 |
| B. | 2 | 4–6 | 48 | 3.53 | 0.83 |
| C. | 16 | 6–22 | 47 | 3.33 | 0.37 |
| II. Data from Engel, et al.** | | | | | |
| D. | 3 | 16–19 | 201 | 4.8 | 1.6 |
| E. | 16 | 0–16 | 113 | 4.8 | 0.8 |

* By analysis of variance, no significant differences were found between the several experimental groups (control, growth hormone treated, adrenal demedullated, and epinephrine treated); the data from these groups were therefore pooled completely. The animals in group A were given saline, those in groups B and C saline or amino acids at 3 hours after nephrectomy.

** Recalculated from Table 1, et seq., of Engel, Pentz, and Engel (2).

for this difference is not apparent, but it may probably be accounted for by such variables as the age of the animals, previous diet, etc. It is evidently not related to the time after operation at which the observations were made. In the present experiments the standard deviations were consistently lower then those of Engel, et al., even where the periods of observations were shorter; from the data in Table 1, it may be calculated that one-half or less the numbers of observations would be required here to obtain the same standard errors as seen in the data of the previous authors. This difference in precision may have resulted in part from the use of more recently operated animals having lower initial levels of blood urea, thus allowing the measurement of larger relative changes.

The effects of the intravenous administration of a protein hydrolysate on the formation of blood urea are shown in Tables 2 and 6. During the first hour after the injection of the amino acids, the rate

of urea formation was increased several fold over the rates seen in animals given only a saline infusion. During the second and third hours, however, the rate returned to the base line, the value in no series differing significantly from the first hour rate in the corresponding control animals.

The amounts of urea formed per 100 gm. body weight were calculated by Engel et al. on the assumption that urea is distributed in a volume of body water equal to 63 per cent of the body weight. On this basis, in animals given the same mixture of amino acids as used here, Engel et al. observed an increase over the basal rate of urea

TABLE 2. CHANGES IN BLOOD UREA IN FASTING NEPHRECTOMIZED RATS AFTER THE ADMINISTRATION OF PROTEIN HYDROLYSATE AND OF A GROWTH HORMONE PREPARATION

| | No. of observ. | Increase in blood urea N mg. per cent per hour (with standard error) | |
|---|---|---|---|
| Time after injection of hydrolysate or of saline (3–3½ hours after nephrectomy) | | 1st hour | 2d and 3rd hours |
| 1. Saline injection* | | | |
| A. Untreated rats | 8 | 3.4±0.40 | 3.9±0.15 |
| B. Rats given growth hormone** | 6 | 3.2±0.51 | 3.0±0.35 |
| 2. Amino Acid injection*** | | | |
| A. Untreated rats | 10 | 11.4±0.37 | 3.3±0.27 |
| B. Rats given growth hormone | 8 | 7.2±0.47 | 3.5±0.18 |
| Difference, treated series less untreated series | 18 | −4.2±0.59 | +0.2±0.34 |
| 3. Increase with amino acid injection over saline control series | | | |
| A. Untreated rats | 18 | +8.0±0.53 | −0.6±0.31 |
| B. Rats given growth hormone | 14 | +4.0±0.70 | −0.5±0.37 |

* 0.5 ml. 0.9 per cent sodium chloride was given per 100 gm. body weight intravenously over 1 minute.

** 1 mg. of partially purified growth hormone per 100 gm. body weight was given intraperitoneally 1½ to 2 hours before experiment (1½ to 2 hours after nephrectomy).

*** Casein hydrolysate (Amigen) in 10 per cent solution: 1 ml. (containing 11 mg. N) per 100 gm. body weight was given intravenously during 1 minute.

formation of 4.08 mg. of nitrogen during 3 hours after the administration of 8.4 mg. of nitrogen per 100 gm. body weight. The extra nitrogen accounted for 48.5±13.3[3] per cent of that injected. As shown in Table 3, in the present experiments where 11.1 mg. of nitrogen were injected, the increment in the first hour, similarly calculated was 5.0 mg. of nitrogen per 100 gm., or 45±2.8 per cent of that administered, a figure in excellent agreement with that of Engel et al. However, it may be noted that all of the increment in rate occurred here during the first hour after the injection, and that greater precision is possible in the comparison of treated and untreated groups when the shorter time interval is used.

*The effect of growth hormone on urea formation:* As shown in Table 2, in the animals given the partially purified growth hormone prepa-

[3] The standard error of this figure was calculated by the present authors.

ration used here, the rate of urea formation during the first hour following the injection of saline did not differ from the control rate. In the second and third hours, there may have been a slight fall, since the difference from the corresponding control series is significant ($-0.92 \pm 0.38$ mg. per cent per hour). However, when the hormone-treated group is compared with any of the other groups of animals given saline (Table 6), the difference is much less evident, and analysis of variance of the four groups indicates a probability of nearly 0.2 that the differences between the group means were fortuitous. There is, moreover, no difference between the rate seen in the second and

TABLE 3. THE EFFECTS OF AN ANTERIOR PITUITARY GROWTH HORMONE PREPARATION ON THE METABOLISM OF ADMINISTERED AMINO ACIDS

These figures were calculated from the increases in blood urea seen in the first hour after the administration of amino acids or of saline (given in Table 2), on the assumption that urea is distributed in water equal to 63 per cent of the body weight. All values are given per 100 gm. body weight.

Nitrogen injected, in the form of casein hydrolysate: 11.1 mg. per 100 gm. body weight.

| | Urea N formed (total | N retained (not metabolized) to urea) | | Urea N formed in controls given saline | Extra urea N formed |
|---|---|---|---|---|---|
| | mg./hr | mg./hr | per cent of injected N | mg./hr | mg./hr |
| 1. Untreated animals | $7.2 \pm 0.23$ | $3.9 \pm 0.23$ | $35 \pm 2$ | $2.1 \pm 0.21$ | $5.0 \pm 0.33$ |
| 2. Animals given growth hormone | $4.5 \pm 0.30$ | $6.6 \pm 0.30$ | $60 \pm 3$ | $2.0 \pm 0.32$ | $2.5 \pm 0.54$ |
| $\frac{\text{Treated}}{\text{Untreated}} \times 100$ | $63 \pm 5$* | $169 \pm 12$ | | | $50 \pm 9$ |

* Standard errors of ratios by the form $(s_{a/b})^2 = (a/b)^2((s_a/a)^2 + (s_b/b)^2)$

third hours in the hormone-treated group and the rates seen in the first hour in any of the animals given saline. It may be doubted then that the observed decrease was in this instance a valid one. It is of course possible that larger doses of the hormone or its administration over a longer period would have induced greater changes in the basal rate of protein catabolism.

The first hour observations in the animals given the protein hydrolysate present the chief points of interest in Table 2. Clearly, the growth hormone depressed the rate of increase in blood urea following the administrations of amino acids. The decrease is equally evident whether the absolute rates are compared or whether comparison is made between the net increases in rate of urea formation over the rate seen in the respective controls given saline.

Further calculations of the amounts of urea formed and nitrogen retained (that is, not metabolized to urea) are presented in Table 3. These values are calculated on the assumption that urea is distributed in all the body water, equal in weight to 63 per cent of the body

weight. Engel, et al., have discussed the validity of this assumption. While it may be subject to some error, there is here no reason to believe that the average amount of water in the body differed in the several series of experiments. The comparison of the amounts of urea N formed are therefore nearly as accurate as indicated by the statistical evaluation. The decrease in the amount of urea formed under the influence of the growth hormone amounted here to $37 \pm 5$ per cent. Given the amount of nitrogen injected, the amount of nitrogen retained was calculated; an increase of $69 \pm 12$ per cent was observed under the influence of the growth hormone. This figure is subject to the error of the estimation of the total urea from the average body water content: if an average value of 70 per cent body water had been used, the increase would have been of the order of 100 per cent.

When the total amounts of urea formed and of nitrogen retained are considered, the assumption is implicit that the administered amino acids enter the metabolic pool and do not differ remarkably in their fate from the body amino acids ordinarily being released. Alternatively, it may be considered that the metabolism of the added amino acids is superimposed on a steady basal rate of protein catabolism. In this case, calculation of the extra urea nitrogen formed is of interest. As shown in Table 3, if this is done, the effect of the growth hormone appears to have been to decrease the rate of catabollsm of the administered amino acids by $50 \pm 9$ per cent.

*The effects of growth hormone on the removal of amino nitrogen from the blood:* The effect of growth hormone in depressing the rate of urea formation from administered protein hydrolysate could conceivably have been due to a delay in the removal of the amino acids from the blood. In order to obtain information on this point, estimations of the concentration of amino nitrogen in the blood were made at intervals following the injection of the hydrolysate or of saline. In the latter case there was observed a slight fall in the blood amino nitrogen, which may have resulted from hemodilution. In the animals given amino acids, the mean amino nitrogen level in whole blood, about 11 mg. per cent initially, was found to be near 18 mg. per cent at 15 min. after the injection, 15 mg. per cent at 30 min., and 12 mg. per cent at 60 min. Because of the rapid removal of amino nitrogen from the blood after the injection of the hydrolysate and the relatively small changes to be seen in whole blood amino nitrogen in any case, there were considerable variations in the levels of blood amino nitrogen at any given time. However, the differences from the initial levels at 30 minutes averaged about 20 per cent less in the treated group, the difference between the groups having a probability of a little more than 0.05 of being fortuitous. Evidently, the growth hormone did not delay the removal of amino nitrogen from the blood. Since the figures were in fact suggestive of some enhancement of the uptake of amino acids from the blood, another series of experiments were performed

under conditions which might be expected to make such an effect more readily observable. Intact animals were given the same growth hormone preparation, and the amino nitrogen content of the plasma followed after the intravenous administration of the protein hydrolysate. The results are given in Table 4. In the group of untreated rats were included a few nephrectomized animals, which did not differ from the intact animals in respect to the changes in plasma amino nitrogen. In these series, the increase in plasma amino nitrogen over the initial levels observed at 30 minutes after the injection of hydrolysate was about 25 per cent less in the animals given the growth hormone than in the untreated animals, and this difference was highly significant. The rate of removal of the amino acids from the plasma appears therefore to have been increased by the growth hormone.

TABLE 4. THE EFFECTS OF A GROWTH HORMONE PREPARATION ON THE REMOVAL OF AMINO NITROGEN FROM THE PLASMA OF NORMAL FASTING RATS

1 ml. of 10 per cent casein hydrolysate per 100 gm. body weight was given intravenously immediately after the initial sample was taken.

| | No. of observ. | Plasma amino nitrogen—mg. per cent Initial | Increase over initial | | |
|---|---|---|---|---|---|
| Time after injection of hydrolysate or of saline (3–3½ hours after nephrectomy) | | 0 | 10 min. | 30 min. | 60 min. |
| 1. Untreated rats | 11 | 7.0 ± 0.3 | +13.8 | +7.3 ± 0.51 | +2.1 |
| 2. Rats given growth hormone* | 8 | 5.2 ± 0.3 | +13.0 | +5.3 ± 0.33 | +1.7 |
| Difference | | −1.8 ± 0.40 | | −2.0 ± 0.67 | |
| t | | 4.5 | | 3.0 | |
| p | | ≪ .01 | | < .01 | |

* 1 mg. per 100 gm. body weight of partially purified growth hormone was given intraperitoneally 1½ to 2 hours before the initial observations.

In Table 4, it may also be seen that the growth hormone preparation had noticeably lowered the basal level of amino nitrogen in the plasma 1 to 1½ hours following the administration of the hormone.

*The effects of intravenous amino acids and of growth hormone on the blood glucose and lactate:* In most of the experiments on the nephrectomized rats, observations were made also of changes in blood glucose and lactate which occurred following the administration of protein hydrolysate or of saline. This was done for several reasons. The observations would serve as some indication of the metabolic state of the recently nephrectomized animals. In the event that the initial levels were normal, as proved to be the case, changes occurring after the intravenous administration of large amounts of amino acids might serve in some degree to indicate the amount of gluconeogenesis which would occur in these circumstances. Then, anterior pituitary extracts containing growth hormone have frequently been observed to cause some diminution in the blood glucose in intact rats; this process has been thought to be the result of inhibition of gluconeogenesis from body protein, but whether it would occur when amino acids were also

given has not been determined. Finally, some information was also desired concerning the possibility that the intravenous administration of large amounts of amino acids might constitute a stimulus to the sympathetic system. A summary of the most pertinent results is presented in Table 5.

The administration of the protein hydrolysate was followed by a small elevation of the blood glucose level which persisted for at least 3 hours. The growth hormone preparation lowered the blood sugar initially (first observation $1\frac{1}{2}$ to 2 hours after its administration), and in the animals given saline further depression continued during the next hour. When the hydrolysate was given to growth hormone

TABLE 5. THE EFFECTS OF PROTEIN HYDROLYSATE AND OF A GROWTH HORMONE PREPARATION ON THE BLOOD GLUCOSE AND LACTATE IN NEPHRECTOMIZED RATS*

| | No. obs. | Blood glucose— mg. per cent Initial** | 1 hour | 3 hours | Blood lactate mg. per cent No. obs. | Initial | 1 hour |
|---|---|---|---|---|---|---|---|
| A. Untreated animals | | | | | | | |
| 1. Saline injection | 7 | 91 | −2 ± 2.2 | +8 ± 4.3 | 4 | 8.9 | 9.0 |
| 2. Amino acid injection | 10 | 88 | +17 ± 2.1 | +15 ± 3.0 | 10 | 8.8 | 11.3 |
| B. Animals given growth hormone | | | | | | | |
| 1. Saline injection | 6 | 79 | −13 ± 3.7 | −10 ± 2.5 | 4 | 13.8 | 18.3 |
| 2. Amino acid injection | 8 | 81 | +9 ± 4.1 | −4 ± 2.7 | 8 | 10.3 | 14.1 |

* Treatment as in Table 1.

** 2 to 3 hours after nephrectomy, just before the administration of the saline or hydrolysate.

The mean initial blood glucose for the 17 untreated animals was 89.4 ± 1.8, for the 14 animals given the growth hormone preparation, 80.0 ± 1.7 (determined on tungstic acid filtrates).

treated animals, little further change occurred. Since the hypoglycemic effect of the growth hormone were observed in the absence of any well defined diminution in urea production (in the animals given saline), and was equally evident whether amino acids were given or not, it seems unlikely that it resulted from depression of gluconeogenesis from protein. Recent evidence on the effects of growth hormone in diabetic animals suggests that enhanced secretion of insulin may be the cause of this hypoglycemia.

No significant changes were observed in the blood lactate in any of the experiments. This fact, together with the small changes in blood glucose, appears to indicate that there was no anoxia, massive sympathetic stimulation, or other untoward metabolic reaction to the intravenous administration of sterile protein hydrolysate.

*The effects of adrenal-demedullation and of epinephrine on urea formation:* As Engel et al. have noted, when non-sterile solutions of amino acids have been given intravenously or when any degree of anoxia has occurred, protein catabolism has been found to be increased (Engle, et al., 1946, 1948). The reason for these effects is unknown. Conceivably, the noxious stimuli could activate the adrenal

cortex and so cause increased release of tissue proteins. Epinephrine has been shown to stimulate the adrenal cortex and might be expected to have a similar effect upon nitrogen metabolism if given or secreted in sufficient amounts. Since the intra-venous administration of large quantities of amino acids is not a physiological procedure, it could cause sympathetic stimulation, discharge of the adrenal medulla, and also possibly consequent activation of the cortex. To test whether adrenal medullary activities were playing a part in the responses to the experimental procedures used here, a few observations were made on animals given epinephrine and on previously adrenal demedullated rats. As shown in Table 6, no significant differences from the normal were observed, either in basal rates or in the metabolism of administered amino acids. The dose of epinephrine used here was sufficient to

TABLE 6. THE EFFECTS OF THE ADMINISTRATION OF INSULIN OR EPINEPHRINE AND OF PREVIOUS ADRENAL DEMEDULLATION ON THE INCREASE IN BLOOD UREA IN NEPHRECTOMIZED RATS.

1 ml. of 10 per cent casein hydrolysate or 0.5 ml. 0.9 per cent sodium chloride per 100 gm. body weight given intravenously.

| | No. of observ. | Increase in blood urea N mg. per cent per hour | |
|---|---|---|---|
| Time after injection | | 1st hour | 2d and 3d hours |
| A. Animals given insulin 0.5 units per kg. intraperitoneally, 30 to 60 minutes before saline or amino acid injection | | | |
| 1. Saline injection | 5 | 4.3 ±0.43 | 5.2 ±0.62 |
| 2. Amino acid injection | 5 | 11.3 ±0.72 | 4.1 ±0.51 |
| B. Animals given epinephrine, 0.05 mg. per kg. intramuscularly at time of saline or amino acid injection | | | |
| 1. Saline injection | 6 | 3.3 ±0.55 | 3.2 ±0.50 |
| 2. Amino acid injection | 4 | 10.0 ±0.78 | 4.0 ±0.42 |
| C. Adrenal demedullated animals (3 to 4 weeks post operative) | | | |
| 1. Saline injection | 3 | 3.9 ±0.18 | 4.0 ±0.44 |
| 2. Amino acid injection | 3 | 10.0 ±0.22 | 3.9 ±0.58 |

raise the blood glucose and lactate of normal fasted rats or of nephrectomized animals well above the initial levels, an average increase of 75 mg. per cent in glucose occurring in 1 to $1\frac{1}{2}$ hours and an increase of 25 mg. per cent in blood lactate in about 45 minutes. Evidently this moderately large amount of epinephrine had no effect on the rate of protein catabolism within 3 hours. Since the initial blood glucose and lactate levels offer no indication that marked adrenal activity was continuing following the recent nephrectomy, it is unlikely that the failure to find any effect of epinephrine on the rate of protein catabolism was due to exhaustion of the mechanisms involved. If the administration of epinephrine were to simulate the effects of other noxious stimuli in this respect, either very much larger doses or their action over longer time intervals would be required. The fact that adrenal demedullated rats did not differ from the normal in these experiments also indicates that secretion of epinephrine, if it occurred

in response to the intravenous administration of saline or amino acids, could not normally have been influencing the results obtained.

*The effects of insulin on urea formation:* Insulin has been observed to increase nitrogen retention in normal animals and to diminish the rate of nitrogen excretion in fasting diabetic animals Also when insulin is given in quantity to eviscerated animals, it will partially suppress the release of amino acids from the tissues (Mirsky, 1938; Frame and Russell, 1946). Mirsky (1939) has in fact suggested that the nitrogen-sparing effects of the anterior pituitary growth factor may be mediated by an "insulinotrophic" action. In the present observations and in other unpublished experiments, the growth hormone has been seen to lower the blood sugar levels in rats in which the pancreas is intact, but not in rats which have been rendered diabetic by alloxan. In order to determine whether additional insulin secretion could have been a factor in reducing urea formation in the present experiments, a series of observations were made in nephrectomized rats given a moderately large dose of insulin. The insulin, 0.5 units per kg., was given intraperitoneally 30 to 50 minutes before the injection of the hydrolysate or of salt solution. The average blood glucose levels seen in the 10 rats so treated were 35, 36, and 61 mg. per cent (true sugar values) respectively at 0, 1, and 3 hours after the observations were begun. As shown in Table 6, no effect of this dose of insulin was seen either on the basal rate of urea formation or on that after the administration of the amino acid mixture. Since in the animals treated with insulin, the blood glucose remained lower throughout the period of observation than it did when the growth hormone was given, (about 60 to 65 mg. per cent true sugar values), it seems unlikely that the effects of the growth hormone were mediated solely through increased insulin secretion in these experiments.

## DISCUSSION

The observations presented above indicate an acute effect of the growth hormone on the metabolism of administered protein hydrolysate. The rate of breakdown of the amino acids was decreased, and since the rate of removal of the amino acids from the blood was not diminished, but probably was increased somewhat, there must have occurred an increase in the retention of the administered amino acids in the tissues. Under the same conditions, little if any effect of the growth hormone was seen on the basal rate of protein catabolism. It may be suggested therefore that the growth hormone here had its most marked effect upon the anabolic phase of protein metabolism.

As to the form in which the administered amino acids were retained in the tissues under the influence of the growth hormone, no information is available from the present experiments. Earlier reports on the effects of crude anterior pituitary extracts in fasting animals indicate that the content of free amino acids (extractable with dilute

trichloracetic acid) and of peptide nitrogen in the tissues was reduced at the same time as the blood levels of amino nitrogen and urea were diminished (Schaffer and Lee, 1935). It seems likely then that the effect of the growth hormone would be to promote the formation of intracellular protein. Whether all tissues take part in this formation of protein, or whether either the liver or peripheral tissues predominate in this process, is still unknown.

The present observations indicate that extra secretion of insulin under the influence of the growth hormone is not alone responsible for the effects of the latter substance on nitrogen metabolism. However, it is still possible that some insulin may be required for the maximal effect of the growth hormone in effecting nitrogen retention. Further investigations of the relationship of insulin to the effects of the growth hormone on nitrogen metabolism are being undertaken.

## SUMMARY

The rate of increase in the blood urea of nephrectomized rats was utilized as a measure of the rate of protein catabolism under basal conditions and after the intravenous administration of amino acids in the form of a sterile solution of casein hydrolysate. When a partially purified preparation of anterior pituitary growth hormone was given to nephrectomized rats 1 to 2 hours before the periods of observation were begun, the rate of urea formation during the first hour after the administration of casein hydrolysate was reduced by approximately 40 per cent and the amount of nitrogen not catabolized to urea increased by about 70 per cent. No effect of the growth hormone on the basal rate of urea formation was seen during the same time interval in animals not given exogenous amino acids.

The rate of removal of amino acids from the plasma of rats given casein hydrolysate was somewhat increased by the growth hormone.

Neither the administration of insulin (0.5 units per kg.) or of epinephrine (0.05 mg. per kg.), nor previous adrenal-demedullation affected the rate of urea formation in the basal state or after the administration of casein hydrolysate.

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# INVOLUTION OF THE ADRENAL CORTEX OF RATS TREATED WITH THIOURACIL

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THERE is considerable contradiction in the literature regarding the influence of antithyroidal drugs on the adrenal cortex. Kennedy and Purvis (1941) reported adrenal hypertrophy after feeding Brassica seed, while Richter and Clisby (1942), Williams, Bissell, Jandorf, and Peters (1944), and Gordon, Goldsmith and Charipper (1944) found no effect on adrenal size. Others have expressed the opinion that any change in the weight of the adrenal gland is concurrent with a loss in body weight and have reported that if the weight of the adrenal gland is expressed as a percentage of body weight no effect is apparent (Leblond and Hoff, 1944; Williams, Weinglass, Bissell and Peters, 1944; Leathem, 1945 and 1946; Smithcors, 1945). Finally, a third group of investigators have reported a significant involution of the adrenal glands as the result of treatment with the goitrogens (Bauman and Marine, 1945; McQuillan and Trikojus, 1946; Deane and Greep, 1947), and have found hemorrhage and necrosis in the adrenal cortex (Endicott, Kornberg and Daft, 1944; Glock, 1945; Daft, Kornberg, Ashburn, and Sebrell, 1946) and loss of cytoplasmic granules (Dalton, Morris and Dubnick, 1945; Deane and Greep, 1947).

The present study was undertaken to re-investigate the question of the action of thiouracil on the adrenal cortex and to determine whether the morphological changes induced by this drug might be correlated with changes in the physiology of the gland (Tepperman, Engle and Long, 1944). Therefore, in addition to obtaining organ weights, it was decided to test the resistance of rats to cold after treatment with thiouracil for varying lengths of time. Furthermore, both immature and mature rats were included in order to determine whether the adrenal changes were due to an inhibition of growth or represented true atrophy.

## MATERIALS AND METHODS

Approximately 750 rats of the Wistar Strain, inbred at the Harvard Biological Laboratories for more than 10 years, were used in the present study. The animals received a standard commercial diet and were kept at a constant temperature in an air-conditioned room. The rats received the thioura-

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TABLE 1. CHANGES IN THE RESISTANCE TO COLD AND THE SIZE OF THE ADRENAL GLAND OF THE FEMALE RAT WITH INCREASING AGE

| No of rats | Age weeks | Body Wt.—Grams | | | Thyroid wt. mgm. | Adrenal wt. mgm. | Adrenal wt./body wt. ×100 | Length of life in cold (hours) |
|---|---|---|---|---|---|---|---|---|
| | | Start | End | Gain | | | | |
| No cold | | | | | | | | |
| 22 | 3 | 36.0 ±0.4* | | | 4.6 ±0.1 | 11.4 ±0.3 | 31.6 +0.8 | |
| 17 | 4 | 35.0 ±0.7 | 61.1 ±1.3 | 26.1 ±0.8 | 6.5 ±0.2 | 15.6 ±0.4 | 25.6 ±0.5 | |
| 20 | 5 | 35.9 ±0.4 | 86.3 ±1.4 | 50.4 ±1.3 | 8.7 ±0.3 | 21.7 ±0.8 | 25.1 ±0.8 | |
| 21 | 7 | 35.4 ±0.6 | 122.2 ±2.7 | 86.8 ±2.7 | 10.3 ±0.3 | 31.1 ±1.1 | 25.5 ±0.7 | |
| 20 | 9 | 34.5 ±0.7 | 150.7 ±3.9 | 116.2 ±4.3 | 10.7 ±0.4 | 38.2 ±2.3 | 25.1 ±1.1 | |
| 18 | 11 | 34.7 ±0.8 | 176.1 ±2.3 | 141.4 ±2.4 | 12.8 ±0.3 | 54.0 ±1.9 | 30.6 ±1.0 | |
| 17 | 13 | 36.0 ±0.7 | 193.7 ±3.3 | 157.7 ±3.1 | 12.4 ±0.5 | 52.8 ±1.9 | 27.4 ±1.0 | |
| 18 | 15 | 34.8 ±0.8 | 207.0 ±2.7 | 172.2 ±2.5 | 15.6 ±0.6 | 58.0 ±2.2 | 28.0 ±1.0 | |
| (Exposed to Cold (3±1°C.) | | | | | | | | |
| 18 | 3 | 35.6 ±0.7 | | | 4.9 ±0.1 | 10.1 ±0.2 | 29.1 ±1.3 | 7.2 ±0.7 |
| 18 | 4 | 38.0 ±0.7 | 58.6 ±1.1 | 20.6 ±1.4 | 5.0 ±0.1 | 10.3 ±0.6 | 27.9 ±1.9 | 21.2 ±1.4 |
| 28 | 5 | 34.4 ±0.6 | 83.1 ±1.5 | 48.7 ±1.0 | 6.0 ±0.1 | 20.2 ±0.6 | 24.2 ±0.5 | 22.6 ±1.8 |
| 20 | 7 | 35.1 ±0.6 | 131.3 ±3.6 | 96.2 ±2.6 | 8.7 ±0.4 | 39.7 ±1.6 | 30.2 ±1.0 | 44.3 ±3.6 |
| 20 | 9 | 36.3 ±0.6 | 156.5 ±3.5 | 120.2 ±3.2 | 10.7 ±0.5 | 51.0 ±2.4 | 32.4 ±1.0 | 42.8 ±2.2 |
| 19 | 11 | 33.6 ±0.6 | 189.7 ±3.4 | 156.1 ±3.2 | 12.5 ±0.5 | 69.6 ±3.8 | 36.5 ±1.7 | 57.2 ±4.1 |
| 17 | 13 | 34.1 ±0.7 | 198.8 ±3.4 | 164.7 ±3.1 | 12.1 ±0.6 | 61.3 ±2.8 | 32.0 ±1.1 | 74.4 ±6.7 |
| 18 | 15 | 34.5 ±0.7 | 193.6 ±2.7 | 159.1 ±2.6 | 13.6 ±0.7 | 67.1 ±1.5 | 34.7 ±0.9 | 66.2 ±5.1 |

* Standard error—Se $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

cil[2] in their drinking water as a 0.1% solution, which was freshly prepared several times a week.

In the cold-exposure experiments the animals were placed in individual wire cages to prevent huddling and put in a cold room which was maintained at 2 to 4°C. The rats were deprived of food and water during the time of exposure to cold and were examined hourly. Body weights were obtained prior to exposure, and at death the thyroids and adrenals were removed and weighed immediately on a torsion balance. The rats referred to as cretins were born from mothers treated with thiouracil for 2 weeks prior to mating and also throughout pregnancy (Hughes 1942). These animals were weaned at 4 weeks of age and continued on the drug for the duration of the study.

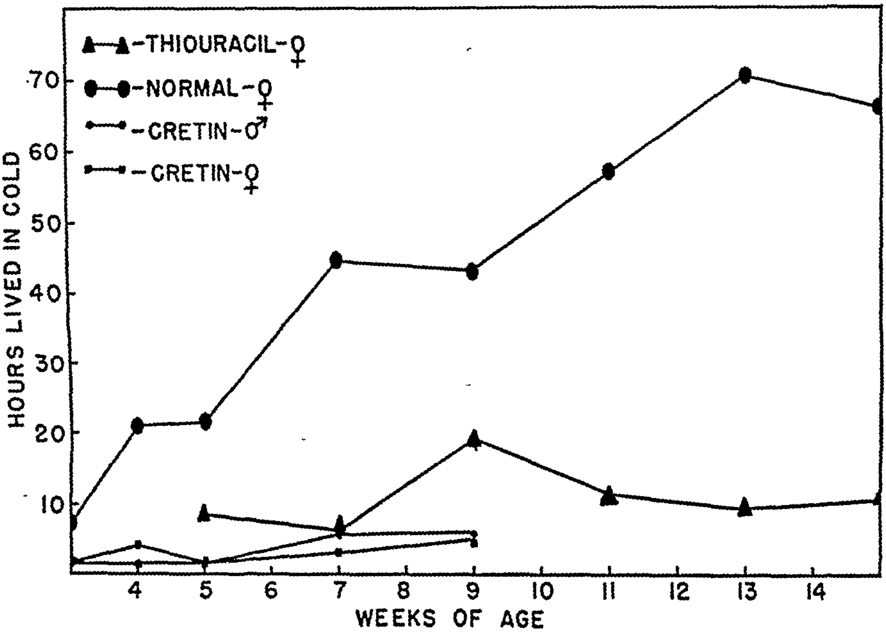


FIG. 1. Resistance of the normal, thiouracil treated, and thiouracil treated cretinoid rat to cold (2° to 4°C.) at different age levels.

Except for the experiments with adult animals and cretins, all the rats were placed on thiouracil at 3 weeks of age. The mature rats weighed approximately 200 grams (4–5 months of age) when placed on thiouracil treatment.

Statistical analyses of the data were carried out according to Snedecor (1946) for correlation coefficients and standard errors. P values were obtained from the tables of Fisher and Yates (1943).

## EXPERIMENTS AND RESULTS

### *Normal Rats*

Groups of normal female rats were exposed to a temperature of $3 \pm 1$°C. at 3, 4, 5, 7, 9, 11, 13 and 15 weeks of age. Similar untreated

[2] We should like to thank Dr. Stanton Hardy, Lederle Laboratories, Pearl River, New York, for the thiouracil used in this study.

control groups were sacrificed at the same time without exposure to cold. Body, adrenal and thyroid weights and length of life in the cold were obtained (Table 1). It was found that the ability of the animals to resist cold increased with age (Fig. 1). It is also interesting to note that no adrenal enlargement due to the stimulation of cold was obtained until the animals were 7 weeks of age, at which time the cold exposed rats had adrenals weighing 39.7±1.6 mgms. and the controls 31.1±1.1 mgms. (Table 1). This difference is also evident if the adrenal weights are expressed as a percentage of body weight. In Figures 2 and 3 it can be seen that the difference in adrenal weights of the controls and cold exposed rats, both as an absolute figure and as a percentage of body weight, is apparent at the 7th week and from that time on the adrenals of the cold exposed rats were persistently heavier. In an attempt to demonstrate a relationship between the size of the adrenal and resistance to cold, adrenal weights were plotted

TABLE 2. CORRELATION VALUES AND SIGNIFICANCE OF PROBABILITY FOR RELATIONSHIP OF LENGTH OF LIFE IN THE COLD AND ADRENAL WEIGHTS

| Age weeks | Treatment | r Value | P Value |
|---|---|---|---|
| 5 | None | ±0.663 | > .001 |
| 9 | None | ±0.486 | 0.02 |
| 11 | None | ±0.173 | <0.1 |
| 5 | Thiouracil | ±0.887 | > .001 |
| 9 | Thiouracil | ±0.616 | > .001 |
| 11 | Thiouracil | ±0.926 | > .001 |
| 9 | Cretin Females | ±0.922 | > .001 |

against the length of life in the cold (Fig. 4). A significant correlation with a P value of less than 0.001 (Table 2) was obtained for the group at 5 weeks of age while no significant correlation was obtained for the older groups.

*Thiouracil Treated Rats*

The rats used in these experiments were started on 0.1% thiouracil in the drinking water at 3 weeks of age, i.e. the time of weaning. The animals in each experiment were divided into 2 groups, of which one group was exposed to cold (3±1°C.) and the other sacrificed at the same time without exposure to cold. The rats were placed in the cold room at 5, 7, 9, 11, 13 and 15 weeks of age, i.e. 2, 4, 6, 8, 10 and 12 weeks after the start of the thiouracil treatment. Body, adrenal, and thyroid weights of all animals were obtained and also the length of life in the cold for the exposed group (Table 3). The data indicate a lack of correlation between resistance to cold and age. Animals 5 weeks of age lived on the average of 8.4±0.9 hours in the cold and those 15 weeks old lived 10.7±1.6 hours; a difference that is definitely not significant. Comparison of the thiouracil treated animals with normal animals indicates that, while the latter show an expected

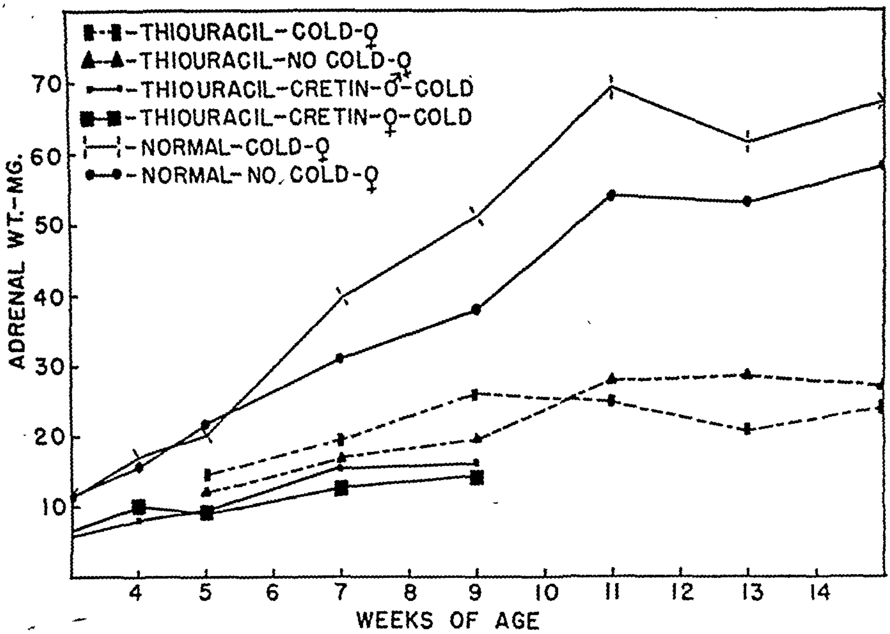


FIG. 2. Changes in the size of the adrenal gland of normal and thiouracil treated rats under normal conditions and after exposure to cold.

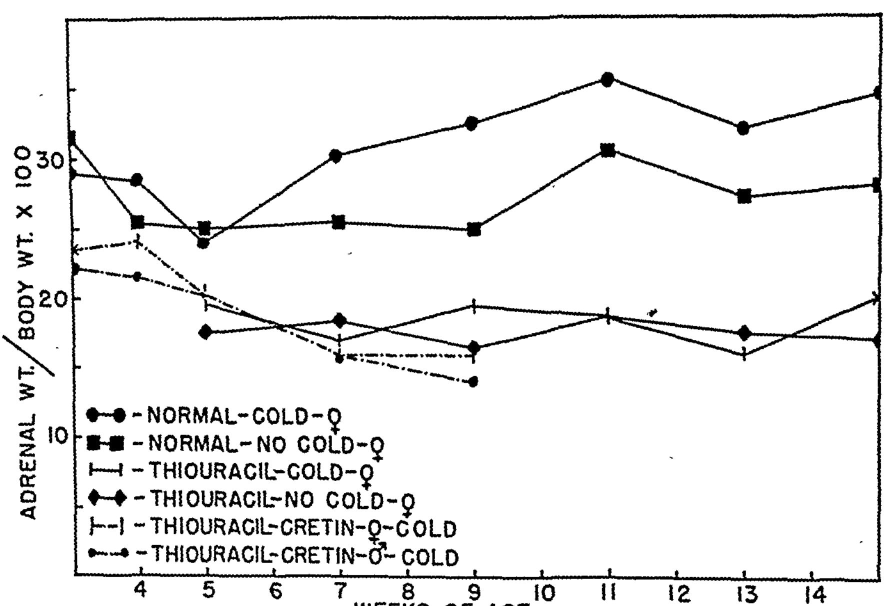


FIG. 3. Changes in the adrenal weights expressed as a percentage of the body weight in the normal and thiouracil treated rat under normal conditions and after exposure to cold.

TABLE 3. CHANGES IN THE RESISTANCE TO COLD AND GROWTH OF THE ADRENAL GLAND OF IMMATURE FEMALE RATS TREATED WITH THIOURACIL

| No. of rats | Age weeks | Body Wt.—Grams | | | Thyroid wt. mgm. | Adrenal wt. mgm. | Adrenal wt./body wt. ×100 | Length of life in cold (hours) |
|---|---|---|---|---|---|---|---|---|
| | | Start | End | Gain | | | | |
| Thiouracil for 2 weeks—No cold | | | | | | | | |
| 25 | 5 | 34.1 ±0.6* | 70.9 ±2.1 | 36.8 ±1.7 | 29.7 ±1.1 | 12.4 ±0.4 | 17.6 ±0.4 | — |
| Thiouracil for 4 weeks—No cold | | | | | | | | |
| 20 | 7 | 36.1 ±0.7 | 95.2 ±3.6 | 59.1 ±3.3 | 50.3 ±3.6 | 17.2 ±0.7 | 18.4 ±0.8 | — |
| Thiouracil for 6 weeks—No cold | | | | | | | | |
| 19 | 9 | 35.6 ±0.7 | 117.5 ±3.8 | 81.9 ±3.7 | 54.6 ±2.3 | 19.6 ±0.8 | 16.7 ±0.5 | — |
| Thiouracil for 8 weeks—No cold | | | | | | | | |
| 20 | 11 | 34.9 ±0.7 | 150.0 ±4.4 | 115.1 ±3.9 | 54.5 ±3.3 | 28.3 ±1.3 | 18.9 ±0.6 | — |
| Thiouracil for 10 weeks—No cold | | | | | | | | |
| 20 | 13 | 36.7 ±0.4 | 165.5 ±3.4 | 128.8 ±3.1 | 58.2 ±3.6 | 28.7 ±1.7 | 17.7 ±0.8 | — |
| Thiouracil for 12 weeks—No cold | | | | | | | | |
| 18 | 15 | 35.6 ±0.7 | 156.5 ±4.6 | 120.9 ±4.7 | 60.4 ±4.5 | 26.6 ±1.9 | 16.8 ±0.8 | — |
| Thiouracil for 2 weeks—Cold | | | | | | | | |
| 40 | 5 | 36.7 ±0.4 | 75.9 ±1.1 | 39.2 ±1.3 | 27.6 ±3.6 | 14.8 ±0.1 | 19.4 ±0.6 | 8.4 ±0.9 |
| Thiouracil for 3 weeks—Cold | | | | | | | | |
| 20 | 7 | 36.5 ±0.5 | 114.3 ±1.7 | 77.8 ±1.7 | 46.1 ±2.6 | 19.5 ±0.6 | 17.1 ±0.5 | 6.2 ±0.8 |
| Thiouracil for 6 weeks—Cold | | | | | | | | |
| 30 | 9 | 35.7 ±0.7 | 130.2 ±3.8 | 94.6 ±3.9 | 47.3 ±3.9 | 26.0 ±1.7 | 19.4 ±1.0 | 19.0 ±3.0 |
| Thiouracil for 8 weeks—Cold | | | | | | | | |
| 19 | 11 | 36.5 ±0.6 | 130.2 ±5.2 | 93.7 ±4.9 | 55.6 ±3.6 | 25.2 ±2.2 | 19.2 ±1.0 | 11.2 ±2.0 |
| Thiouracil for 10 weeks—Cold | | | | | | | | |
| 19 | 13 | 36.7 ±0.6 | 126.9 ±7.3 | 90.2 ±7.5 | 62.4 ±2.3 | 21.0 ±2.2 | 16.0 ±0.8 | 9.8 ±2.0 |
| Thiouracil for 12 weeks—Cold | | | | | | | | |
| 17 | 15 | 35.5 ±0.7 | 122.1 ±7.2 | 86.6 ±6.9 | 52.9 ±4.2 | 24.2 ±1.6 | 19.9 ±0.6 | 10.7 ±1.6 |

* Standard error.

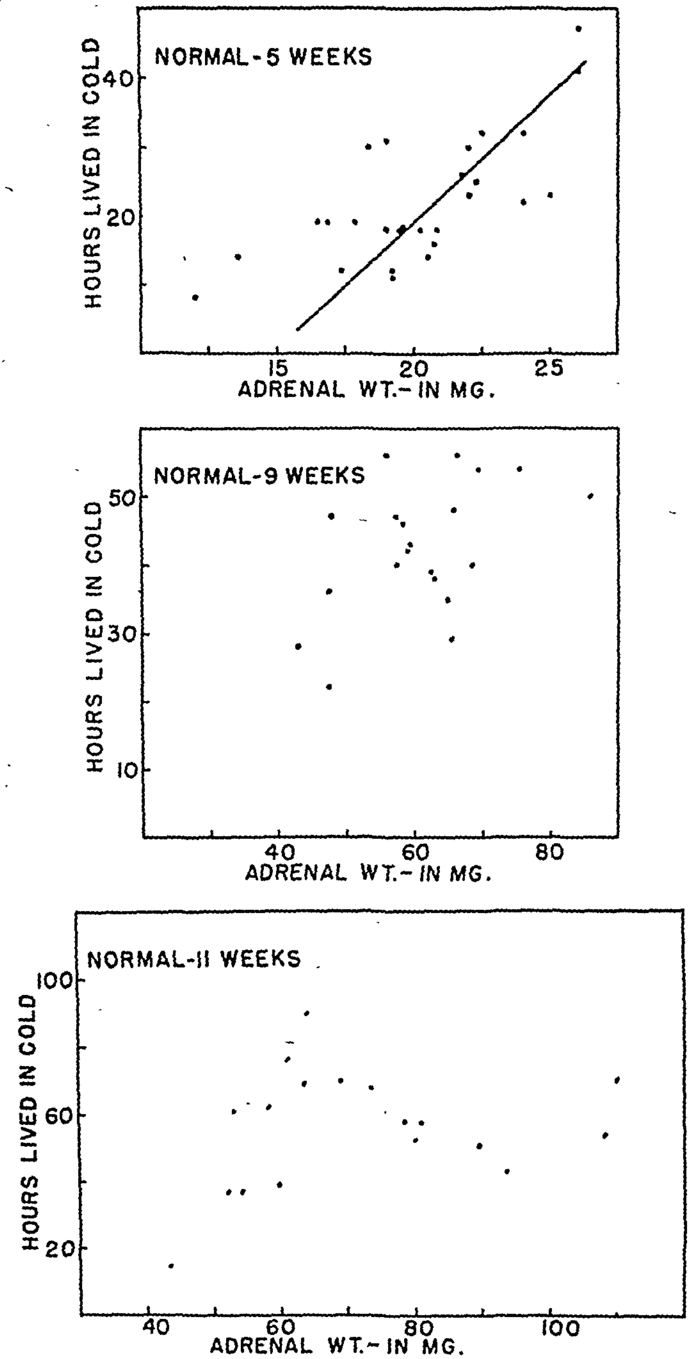


FIG. 4. Relationship between the weight of the adrenal gland of the normal female rat and its ability to withstand cold.

*Adult Rats Treated with Thiouracil*

Experiments were carried out in which adult female rats were divided into 4 groups and treated in the following manner: 1) exposure to cold after 10 weeks of thiouracil treatment, 2) controls given thiouracil for 10 weeks, 3) normal animals exposed to cold and 4) normal animals not exposed to cold.

The data indicate that the resistance to cold in the thiouracil treated animals was markedly decreased (Table 4). The normal control rats lived an average of 61.9±5.4 hours while the treated rats remained alive for only 22.5±4.8 hours; an unquestionably significant drop in resistance to cold. The data show also that the adrenals of the treated animals decreased in weight. This atrophy is apparent both with regard to the absolute weight of the adrenals and to the weight of the adrenals expressed as a percentage of the body weight. The

TABLE 5. FAILURE OF THE ADRENAL GLAND AND LACK OF RESISTANCE TO COLD IN THE YOUNG CRETIN RAT

| No. of Rats | Age weeks | Body wt. grams | Thyroid wt. mgm. | Adrenal wt. mgm. | Adrenal wt./body wt.×100 | Length of life in the cold (hours) |
|---|---|---|---|---|---|---|
| | | | Males | | | |
| 9 | 3 | 28.4 ±2.1* | 12.7 ±1.73 | 6.2 ±0.37 | 22.3 ±1.23 | 1.3 ±0.115 |
| 14 | 4 | 38.4 ±1.54 | 21.6 ±1.63 | 8.3 ±0.397 | 21.6 ±0.064 | 1.4 ±0.0826 |
| 6 | 5 | 46.5 ±3.4 | 35.3 ±6.48 | 9.5 ±0.956 | 20.6 ±1.49 | 2.1 ±0.375 |
| 10 | 7 | 96.7 ±5.7 | 50.6 ±3.28 | 15.4 ±1.068 | 16.1 ±0.958 | 6.1 ±1.51 |
| 6 | 9 | 113.5 ±10.5 | 50.2 ±6.19 | 16.0 ±1.98 | 14.1 ±0.834 | 6.2 ±1.495 |
| | | | Females | | | |
| 7 | 3 | 28.4 ±2.52 | 14.7 ±2.17 | 6.6 ±0.514 | 23.7 ±1.51 | 1.4 ±0.211 |
| 23 | 4 | 37.3 ±1.86 | 21.7 ±1.649 | 10.0 ±0.71 | 24.3 ±1.055 | 3.9 ±1.137 |
| 12 | 5 | 46.8 ±3.235 | 25.2 ±2.92 | 9.2 ±0.569 | 20.5 ±1.369 | 1.9 ±0.214 |
| 9 | 7 | 80.0 ±5.3 | 42.4 ±4.86 | 12.8 ±1.23 | 16.2 ±1.39 | 3.0 ±0.593 |
| 15 | 9 | 89.5 ±8.1 | 48.1 ±2.84 | 14.6 ±1.49 | 16.5 ±0.84 | 5.4 ±1.415 |

* Standard error.

absolute adrenal weight in the normal rat was 60.5±2.4 mgm. while the thiouracil rats had adrenals with a weight of 44.2±2.5 mgm. Exposure to cold caused an increase in the adrenal weight of the normal rats (78.3±4.6 mgm.) whereas no significant change was noted in the adrenals of the thiouracil treated rats (45.5±2.4 mgms.). These differences are also apparent if the adrenal weights are expressed as a percentage of the body weight. As was expected the controls gained 34.7 grams in body weight whereas the treated animals gained only 19.3 grams; nevertheless, the atrophic changes in the adrenals were relatively much greater.

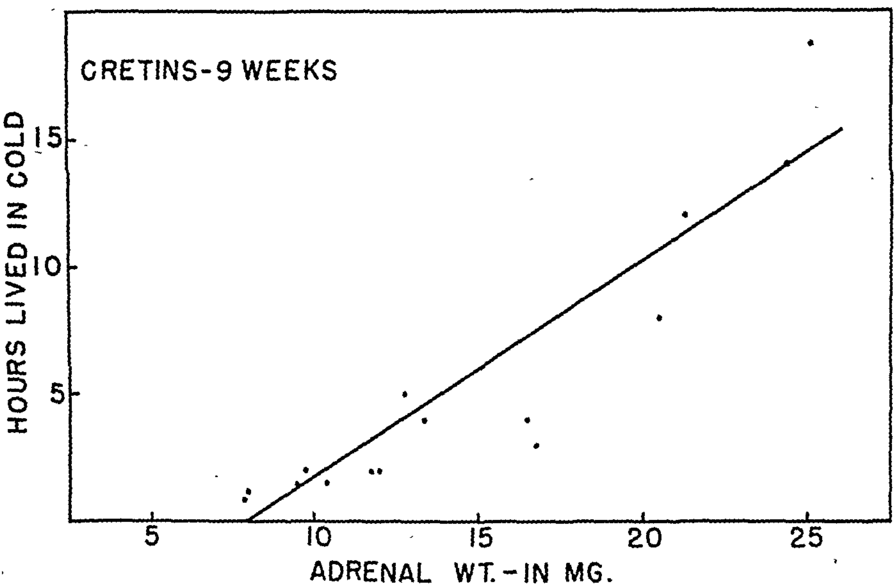


FIG. 6. Correlation between the size of the adrenal gland ability to resist cold (2° to 4°) in the thiouracil cretinoid rat. Relationship between the weight of the adrenal gland of the cretinoid rat and its ability to withstand cold.

*Cretinoid Rats*

Studies were carried out on a group of young cretinoid rats of both sexes. These animals were obtained from mothers that had been on thiouracil treatment for at least two weeks prior to mating and kept on thiouracil until the young were weaned. After weaning, the young were continued on thiouracil for the duration of the experiment. All litters were kept with the mother for 4 weeks in place of the usual 3 weeks in an attempt to keep the size of the young rats constant. However, in spite of this precaution large variations in weight occurred.

These animals (Table 5) showed no increase in their ability to resist cold as they grew older. They behaved in a manner similar to that found for rats started on thiouracil at the time of weaning both

with regard to their resistance to the cold (Fig. 1) and size of their adrenals (Fig. 2). The weights of the adrenals of cretinoid rats that had been exposed to cold were significantly less than those for the normal controls. As noted with the other thiouracil treated rats the cretinoids also showed a definite relationship between length of life in the cold and adrenal weights (Fig. 6) and a significant correlation for the fit of the data (Table 2).

## DISCUSSION

It is apparent from the present data that definite morphological changes take place in the adrenal cortex of rats placed on thiouracil treatment. Although a contradictory literature exists with regard to this point, as was indicated in the Introduction, part of the contradiction may be due to the length of treatment. Our results indicate that it is necessary to keep the rats on thiouracil for more than 2 weeks before definite atrophic changes may be observed in the adrenal cortex. It has also been argued that any changes in the adrenal cortex are merely a reflection of loss in body weight (Leblond and Hoff, 1944; Leathem, 1945). In the present investigation though a loss in weight was encountered, nevertheless, the atrophic changes in the adrenal gland were far greater. Thus, when the adrenal weights were expressed as a percentage of the body weight, a highly significant decrease in the weight of the adrenal cortex was still found.

The loss in the weight of the adrenal cortex was obtained following treatment with thiouracil when: 1) treatment was started in the mother prior to pregnancy and continued in the young, 2) treatment was started at 3 weeks of age and 3) treatment was started after the rats had reached maturity. We may thus conclude that thiouracil not only inhibits the growth but also produces a true atrophy of the adrenal cortex.

These results are not too surprising in view of the early work of Hoskins (1910) and confirmed by Gardner (1942) that thyroid feeding causes adrenal hypertrophy and the work of Zeckwer (1938) indicating that thyroidectomy caused a loss in the weight of the adrenal cortex of the female rat. If one considers thiouracil treatment in the simplest concept as a chemical thyroidectomy one might expect adrenal involution.

The complementary action of the adrenal cortex and the thyroid in protection against cold has been apparent for some time. Such a relationship has been shown for other activities also. In 1934 Koelsche suggested a thyroid-adrenal cortex interrelationship with respect to nitrogen balance. This has been confirmed by Wells and Chapman (1940) while White and Dougherty (1947) suggest that adrenal cortical secretion has an augmenting influence on thyroid activity. Thus it would appear that a complementary thyroid-adrenal relationship

exists with regard to several activities and that with respect to cold protection antithyroidal drugs inhibit both the thyroid and the adrenal cortex.

It has been shown by numerous investigators that the adrenal cortex will protect animals against cold exposure. This procedure has been used for the assay of cortin (Selye and Schenker, 1938), DCA (Zarrow, 1942) and 11-oxycorticosteroids (Dorfman, Shipley, Schiller, and Horwitt, 1946). The latter have also shown that the 11-oxycorticosteroids are far more active than the DCA compound in protection against cold. Furthermore, Deane and Greep (1947) have indicated that in hypothyroidism, due either to thyroidectomy or thiouracil treatment, the steroid content of the adrenal as shown by histochemical techniques decreases markedly. The present results indicate that the morphological involution of the adrenal is accompanied by a physiological involution as shown by the marked decrease in the ability of the rat to withstand cold after thiouracil treatment. Ershoff (1948) has also indicated a decreased resistance in the rat after treatment with antithyroidal drugs. In view of the work of Deane and Greep (1947) it would appear that thiouracil treatment disturbs the formation of the 11-oxycorticosteroids.

It is of further interest to note that a definite relationship was obtained between the weight of the adrenal glands of thiouracil treated rats and their resistance to cold. In the normal group this relationship was found only in the 5 week old rats and not in the older age groups. This may indicate that when the animal gets older its reserve of adrenal tissue is much greater than its immediate needs, and consequently a relationship between adrenal size and function is apparent only in the normal young rat or in the thiouracil treated rat. In the latter instance, the resulting involution has probably been sufficient to diminish the reserve and thus bring out the relationship.

Failure to obtain adrenal hypertrophy in the thiouracil treated rats cannot be regarded as due to an insufficient length of exposure. Adult rats treated with the drug lived in the cold for a period of $22.5 \pm 4.8$ hours and it has been shown that 12 hours of exposure to stress is sufficient to cause adrenal hypertrophy (Ingle, 1938). It would appear then that this lack of response might be due either to a lack of ACTH from the pituitary or an inability of the adrenal to react to the available ACTH.

## SUMMARY AND CONCLUSIONS

Treatment of young female rats with thiouracil for 6 weeks or longer results in an atrophy of the adrenal cortex. This change is not due to a loss in body weight of the treated animal for the adrenals are much lighter when expressed either as an absolute figure or as a percentage of the body weight. The same situation occurs also in the

cretinoid rat and in mature animals. Thus one may conclude that the drug not only inhibits the growth of the adrenals but also causes a true involution.

Exposure of thiouracil treated rats to a temperature of $3 \pm 1°$C. indicates an unquestionable decrease in the ability of the animal to withstand cold. Thus the involution of the adrenal cortex after thiouracil treatment is both of a morphological and physiological nature. Within single age groups of thiouracil treated rats, a definite and constant relationship is noted between the size of the adrenal cortex and the resistance to cold. This relationship is found to exist only in the 5 week old group among normal rats. No adrenal hypertrophy occurs in the thiouracil treated rats after exposure to cold.

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# INFLUENCE OF STEROID HORMONES ON GRANULATION TISSUE

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## INTRODUCTION

Studies concerning the influence of steroid hormones upon connective tissue have, for the most part, been restricted to pelvic or sex organs (Loeb, Suntzeff and Burns, 1939; Gardner and Pfeiffer, 1943; Hall, 1947; Hall and Newton, 1947; Talmage, 1947; Emery and Lawton, 1947). More recently the influence of steroids, particularly desoxycorticosterone acetate, upon the body as a whole with the production of specific or systemic effects has been stressed by Selye (1943, 1944, 1946; Selye, Sylvester, Hall and Leblond, 1944). We desired to study the effects of different steroid hormones on connective tissue in laboratory animals under controlled conditions.

We became interested in one particular phase of this problem, i.e. the response of connective tissue to inflammation under the influence of selected steroid hormones. It was thought that one method would be to observe actively growing granulation tissue in experimental animals. Sterile turpentine abscesses were produced in adult and growing rats and the resultant granulation tissue in their walls studied. A comparison was then made between the response of the granulation tissue in normal animals and those which were under the influence of desoxycorticosterone acetate, testosterone propionate, or estradiol dipropionate in excess. Another series of animals were rendered comparatively free of circulating steroid hormones by the extirpation of the adrenals and gonads, and their responses were observed.

## METHODS[2]

Two series of rats of the Sprague-Dawley strain were employed. The first consisted of male adults each weighing 200 grams and the second of young males and females 40 to 50 grams in weight. Nine adults were employed in each of the following groups: (1) controls, (2) those receiving 1 mgm. of

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desoxycorticosterone acetate, (3) animals receiving 2.5 mgm. of testosterone propionate, (4) animals receiving 1 mgm. of estradiol dipropionate, and (5) adrenalectomized and castrated rats. All steroid hormones were administered daily for a period of five weeks. Estradiol dipropionate and testosterone propionate were dissolved in sesame oil, desoxycorticosterone acetate in sesame or peanut oil.

Two groups, each consisting of four animals were given only peanut and sesame oil respectively, before producing abscesses.

The series of young animals, females as well as males, were divided into groups of five rats each, which were treated similarly to the above, excepting that the dosage of desoxycorticosterone acetate was 0.5 mgm., of testosterone propionate 1 mgm., and of estradiol dipropionate 0.5 mgm. Hormones were administered four times a week. Adrenalectomized-castrated animals were not employed in this series.

Using 0.5 cc. of turpentine U.S.P., abscesses were produced in all animals subcutaneously over the right scapular region. These were induced after the hormones or the oil had been administered for five weeks, and in the adrenalectomized-castrated animals three days post-operatively. One or several animals of each group were sacrificed daily beginning on the day of abscess induction.

Immediately upon sacrificing each animal the abscess was excised and fixed in a 4% formaldehyde solution. Histologic preparations were stained with iron hematoxylin and eosin, Masson's trichrome stain for the study of collagen, and Schiff's reagent after treatment of the tissue with periodic acid, for the differentiation of ground substance and "glycogen" containing elements.

The abscess walls were examined in each instance with particular reference to certain features. In order to establish a standard with which to compare the amount of fibroblastic response, a histologic constant was sought. The wall of leukocytes immediately adjacent to the turpentine was of similar thickness in all animals examined. This was designated as "L" and given a figure of one. The thickness of the fibroblastic wall was designated as "F" and used as a numerator in the ratio F/L. In addition, in an attempt to judge the exuberance of the fibroblastic response it was graded from one to five. The appearance of the fibroblasts in the controls on the fourth day of

[3] The diets consisted of Purina Dog Chow Pellets. In addition, adrenalectomized-castrated animals received 0.9% saline solution in place of tap water.

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Figs. 1–5 inclusive, ×160, illustrating granulation tissue in walls of four day old turpentine abscesses. Delimiting zone of necrosis and polymorphonuclear leukocytes on extreme left of each figure. Figs. 1a–5a inclusive, ×640, illustrating types of fibroblasts. All sections stained with iron hematoxylin and eosin.

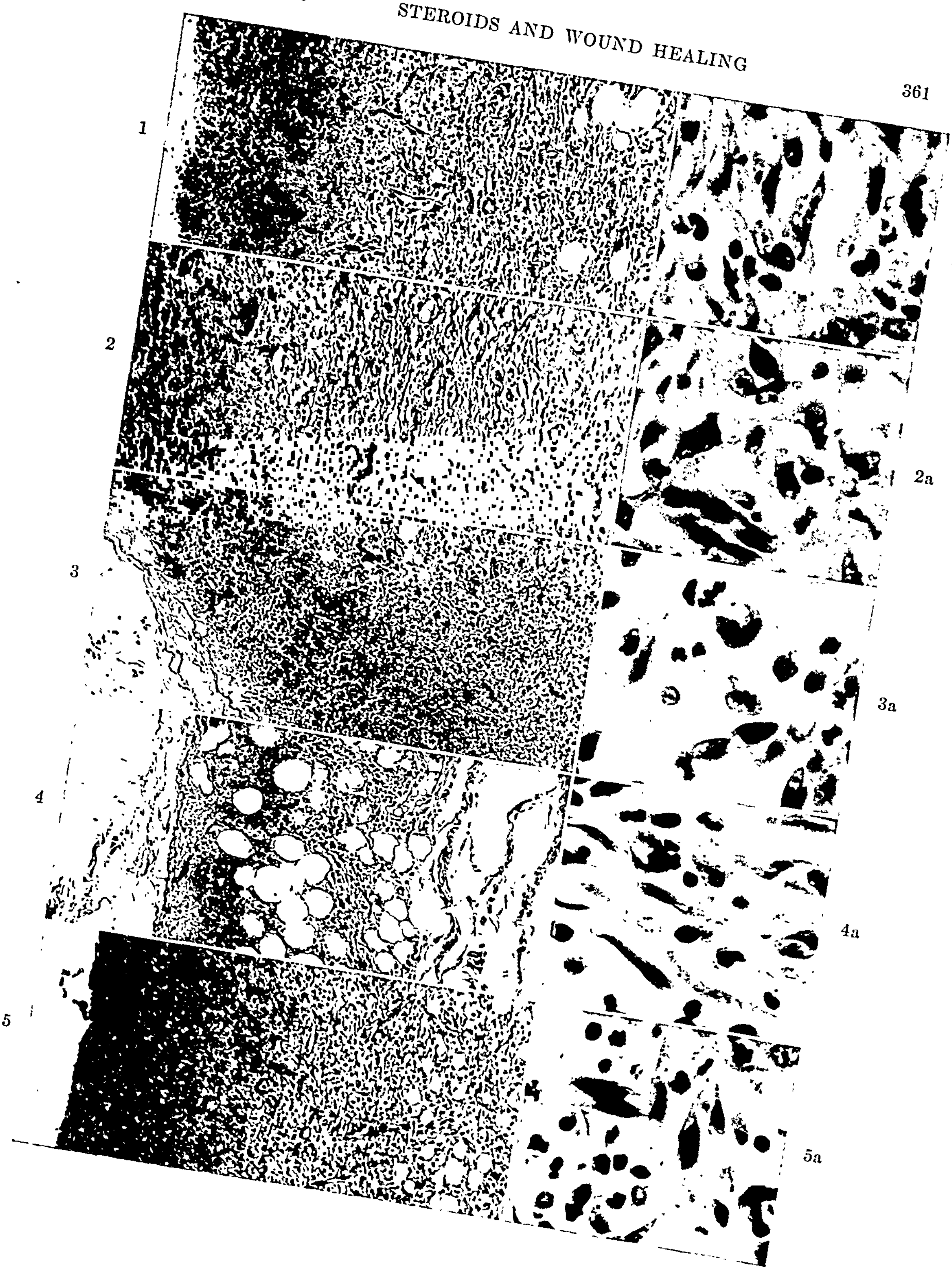
Figs. 1 and 1a. Abscess in control animal.

Figs. 2 and 2a. Abscess in animal receiving excesses of desoxycorticosterone acetate. Note thickness of wall of granulation tissue and large multipolar plump fibroblasts.

Figs. 3 and 3a. Abscess in animal receiving testosterone propionate. Note thinness of fibroblastic wall and sparse, small fibroblasts.

Figs. 4 and 4a. Abscess in animal receiving estradiol dipropionate. Note almost complete absence of granulation tissue and very thin fibrillar fibroblasts.

Figs. 5 and 5a. Abscess in adrenalectomized-castrated animal. Note close similarity to that of control.



1 1a
2 2a
3 3a
4 4a
5 5a

the turpentine abscesses was designated as three. A fibroblastic response of one and two was considered inferior and that of four and five superior to that of the controls. The types and histologic appearance of the fibroblasts were studied. In addition, the ground substance or collagen was noted from the point of view of its type of distribution and staining properties with Masson's trichrome stain and the periodic acid routine. Other factors such as the vascular response and localization of the abscesses were also noted.

## RESULTS

The abscesses, even *grossly*, showed differences. Those animals in the first series which received desoxycorticosterone acetate had thicker abscess walls which were more sharply demarcated than any

TABLE 1. ADULT RATS—SUMMARY OF FOURTH AND FIFTH DAYS AFTER INDUCTION OF TURPENTINE ABSCESSES

| | F/L | Fibroblastic response | Types of fibroblasts | Collagen | Miscellaneous |
|---|---|---|---|---|---|
| Controls | 2 | 3 | Large, with 2 or more processes | Moderate amount in coarse clumps | |
| Desoxycorticosterone acetate | 4–4.5 | 5 | Luxuriant, large, much activity, prominent nucleoli | Diffuse, homogeneous, pale staining | Granulation tissue thick, well demarcated |
| Testosterone propionate | 1–1.5 | 1 | Thin, spindle-shaped, sparse | Very scant. Occasional coarse clumps | Poorly demarcated abscesses |
| Estradiol dipropionate | 0–0.5 | 0–1 | Difficult to find. Thin, flat, small | Usually absent. Occasional delicate strands | Granulation tissue very difficult to find |
| Adrenalectomized-castrated | 2–2.5 | 1–2 | Thinner, smaller nuclei, less cytoplasm | Sparse coarse clumps | |

of the others. In addition, the abscesses in these animals healed faster than in any of the other groups and terminated as zones of fibrosis early. The contrast was greatest grossly as well as microscopically between the desoxycorticosterone group and the rats which had received the sex hormones. The latter animals, particularly those receiving estradiol dipropionate, had abscesses which were so poorly defined that within the first four to five days they could be identified grossly only with greatest difficulty. At the termination of the experimental period, their walls were barely discernible, thin, and very poorly demarcated from the surrounding edematous subcutaneous tissue.

A summary of the microscopic picture of the abscesses in the adult rats is given in Table 1.

The *control group* of turpentine abscesses in both series went through a similar development. During the first two days there was extensive edema, moderate numbers of polymorphonuclear leukocytes and large macrophages filled with foamy material around the central accumulations of turpentine. Granulation tissue, as evidenced by thin-walled capillaries and proliferating fibroblasts was noted as early as the second day. By the third day the F/L ratio was 0.5. There were many large macrophages and the fibroblastic response was graded as

zero to one. Collagen was apparent as concentric coarse blue-staining clumps with Masson's trichrome stain. On the fourth day the F/L ratio was two. The fibroblastic response was graded as three. The cellular elements consisted of a mixture of moderately large and active fibroblasts, many thin-walled capillaries perpendicular to the center of the abscess, and macrophages. Collagen became more abundant, appeared in concentric lamellae, and was often seen in coarse clumps. There followed a gradual increase in the amount of collagen and fibroblasts. The latter increased in number but decreased in thickness and size. They assumed a more mature appearance, became elongated, thin and flattened. These alterations corresponded with an increase in collagen and the appearance of densely staining collagen in thick coarse lamellae. Inflammatory cells, except for the central zone of polymorphonuclear leukocytes and macrophages, disappeared. From the sixth day onward the F/L ratio averaged five. The periodic acid routine with Schiff's reagent added little to the observations. Pink-staining fibers and strands between fibroblasts corresponded roughly with the material staining blue with Masson's trichrome stain. The intercellular pink-staining material apparently was either part of or coincident with the collagen fibers. The only feature not revealed previously by other staining methods was the appearance of pink-staining granules within fibroblasts beginning at about the fifth day in the control group.

The animals receiving only sesame or peanut oil developed turpentine abscesses similar to the above control group.

The animals receiving *desoxycorticosterone acetate* revealed several prominent differences from those of the controls. The most pronounced was the thickness of the granulation tissue. The F/L ratio was four to four and one-half from the fourth to sixth days. The fibroblastic response was extreme. This was graded as four to five. The individual cells were large with very large nucleoli and abundant cytoplasm. Frequent mitoses were seen. These cells had numerous cytoplasmic processes which often gave them a stellate appearance. There was little or no orderly arrangement of the fibroblasts, whereas in the control group they tended to a lamellar arrangement about the central abscess cavity. The appearance of the collagen laid down between these fibroblasts was striking. It distinguished the abscesses of the desoxycorticosterone acetate animals from any of the others. The collagen was very abundant and diffuse. Instead of being arranged in clumps, it had a homogeneous, almost glassy, appearance and stained light blue with Masson's trichrome stain. The delimitation of these abscesses occurred early in their development and was very distinct. Almost complete cicatrization appeared one to two days earlier than in the controls. Capillarization was abundant and profuse.

The following differences appeared in the animals which had received *testosterone propionate* and *estradiol dipropionate*. The abscesses

in both of these were very poorly defined, their walls thin, almost completely void of granulation tissue and consisted almost entirely of polymorphonuclear leukocytes. The F/L ratio in the animals treated with testosterone propionate on the fourth day was one to one and one-half. The fibroblastic response was graded as one; the fibroblasts were sparse, very thin and spindle shaped. Collagen appeared in extremely rare, coarse clumps. The lack of response was more pronounced in rats receiving estradiol dipropionate. The F/L ratio in the first series of animals was zero to one-half. Granulation tissue was often impossible to find and fibroblasts were extremely scarce. When found they were small. Intercellular collagen was very sparse and when encountered was in thin delicate strands.

The first series also contained a group of animals which were castrated and adrenalectomized and thus were deprived of the two chief

TABLE 2. YOUNG GROWING RATS—SUMMARY OF FOURTH AND FIFTH DAYS AFTER INDUCTION OF TURPENTINE ABSCESSES

| | | F/L | Fibroblastic response | Types of fibroblasts | Collagen |
|---|---|---|---|---|---|
| Controls | —Males | 3.5 | 3 | Moderately thick and large, moderately active | Coarse clumps, moderate amount |
| | —Females | 2 | 3 | Moderately thick and large, moderately active but smaller than desoxycorticosterone acetate animals | Occasional fibrillar strands |
| Desoxycorticosterone acetate | —Females | 2.5 | 4 | Large, luxuriant, active, numerous | Diffuse, pale staining |
| Testosterone propionate | —Males | 1–1.5 | 2 | Thin, very sparse | Sparse, in clumps. In some cannot be found |
| | —Females | 2 | 1 | Thin, sparse | Clumps, many very coarse |
| Estradiol dipropionate | —Males | 1.5–2 | 1–2 | Very thin, sparse | Very sparse clumps—often absent |
| | —Females | 0.5–1 | 0–1 | Thin, sparse | Sparse, in occasional thin fibers bers and clumps |

sources of steroid hormones. The F/L ratio was slightly greater than the controls and averaged two and one-half in the four and five day abscesses. The fibroblasts were predominantly of the bipolar variety, tended to be smaller and flatter than those of the controls. In addition, the fibroblastic response was much less evident and was graded between one and two. Collagen was very sparse and appeared only as rare, coarse clumps.

In the series of young animals (Table 2) the response as judged by the F/L ratio was not as pronounced and there was one discrepancy as compared with the adults. The F/L ratio of a male control was three and one-half as compared with two and one-half for that of the desoxycorticosterone acetate. Despite the relatively slight differences in the F/L ratios in the two series of animals, the fibroblastic responses, types of fibroblasts, and type and distribution of collagen were similar in both. Gross changes of some significance were seen in

the animals. The gain in weight of the male controls was much more rapid, and terminally these animals weighed 85 to 100 grams more than the females which had received desoxycorticosterone acetate.

The periodic acid and Schiff's reagent routine added little to the observations. Pink-staining intercellular material paralleled in distribution and amount the collagen fibers as noted with Masson's trichrome stain. In addition, red granules were noted within some fibroblasts in greater amounts within the desoxycorticosterone animals and were practically absent in those treated with sex hormones. Whether this intracellular material was secretion produced by large macrophages or ingested glucoportein from necortic connective tissue was not determined.

Of some interest was the variation in weights of the growing young animals as summarized in Table 3. Injections of testosterone propionate and estradiol dipropionate inhibited the growth of young animals. This was particularly true of the latter hormone.

TABLE 3

| | Weight in grams at start of experiment | | Weight in grams at end of experiment | |
|---|---|---|---|---|
| | *Female* | *Male* | *Female* | *Male* |
| Controls | 40–50 | 40–50 | 150–160 | 200–255 |
| Desoxycorticosterone acetate | 40–50 | 40–50 | 115–150 | |
| Testosterone propionate | 40–50 | 40–50 | 116–130 | 135–160 |
| Estradiol dipropionate | 40–50 | 40–50 | 95–100 | 95–105 |

## DISCUSSION

The characteristic structure of granulation tissue in turpentine abscesses was studied in control animals as well as those receiving excesses of various hormones. Our experiments have shown that the administration of steroid hormones, i.e. desoxycorticosterone acetate, testosterone propionate, and estradiol dipropionate, and the removal of organs producing steroid hormones, have an effect on granulation tissue.

The results were outstanding in two major groups of animals. Those receiving desoxycorticosterone acetate manifested an exuberant fibroblastic response with many giant young fibroblasts and the laying down of a diffuse homogeneous groundwork of collagen. In sharp contrast, animals receiving sex hormones showed an inhibitory influence on granulation tissue with sparse and small fibroblasts and scant collagen clumps. Although of small amount in both, the collagen in the animals receiving estradiol dipropionate was of a finer, more delicate pattern and more sparse. In those receiving testosterone propionate the collagen was in coarser clumps.

Strangely enough, in castrated-adrenalectomized animals the re-

sponse of the granulation tissue was only slightly inferior to the controls.

At the present time we are unable to apply the known physiologic functions of these hormones to our observations. However, two factors must be considered. First, a possible direct influence of the administered hormones upon the proliferating granulation tissue and, second, an indirect influence. Among the latter are alterations in other mechanisms which may influence granulation tissue. In the case of desoxycorticosterone acetate, which profoundly affects water and salt metabolism, it is possible that our results are actually due to electrolytic changes. This is suggested by Selye's observations (1943) that salt-free diets inhibit the proliferation of vascular connective tissue in the kidneys in animals under the influence of desoxycorticosterone acetate. Influx of electrolytes into healing wound territory was reported previously (Andreesen and Tammann, 1933; Tammann, Bluemel and Roese, 1933). Potassium, in particular, seemed to play a major role. The part of the suprarenal cortex in these processes is unknown. Although it has been suggested (Selye, 1947) that wound healing is retarded in adrenalectomized animals and improved by administration of adreno-cortical extracts, our experiments show that even in the absence of suprarenals, granulation tissue is able to grow well.

The influence of sex hormones upon wound healing has been reviewed by Arey (1936). Earlier experiments are inconclusive. Inhibition of the anterior pituitary by excesses of sex hormones may explain the extremely poor response of the granulation tissue in animals receiving testosterone propionate and particularly estradiol dipropionate. This mechanism is suggested in our young animals in which the marked inhibition of growth of some rats receiving the sex hormones may have secondarily altered the histologic characteristics of the granulation tissue. Stunting of growth after administration of large doses of estrogenic hormones has been reported by many observers (Gardner and Pfeiffer, 1943). Doubts were raised as to whether this occurs through inhibition of the pituitary or by direct influence upon the epiphyseal cartilage (Gaarenstrom and Levie, 1939; Griffith and Young, 1942). Work is in progress to determine the exact roles of the above mentioned factors in formation of granulation tissue.

## SUMMARY AND CONCLUSIONS

Granulation tissue in the walls of turpentine abscesses in normal rats as well as those receiving excessive amounts of various steroid hormones was studied. The results were compared with animals rendered relatively void of steroids through adrenalectomy and castration.

Desoxycorticosterone acetate stimulates fibroblasts and encourages the deposition of a homogeneous diffuse groundwork of collagen.

Testosterone propionate inhibits granulation tissue with the production of scant coarse clumps of collagen.

Estradiol dipropionate inhibits fibroblastic response to an even greater degree than testosterone. The collagen fibers are more delicate.

Adrenalectomy and castration of rats results in granulation tissue only slightly inferior in the fibroblastic response, the type, and amount of collagen of the control animals.

The possible factors in the production of these results were discussed.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# THE EFFECTS OF INDUCED HYPER- AND HYPO-THYROIDISM ON THE RESPONSE TO A CONSTANT DOSE OF PREGNANT MARE'S SERUM IN IMMATURE MALE RATS AND MICE[1]

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THE influence of altered levels of thyroid activity on the response to administration of gonadotrophic or gonadal hormones has not been adequately clarified. The reports available to the authors on this subject have been summarized in Table 1. It can be seen that in most instances the response of rats to the injection of gonadotrophic or gonadal hormones was increased following thyroidectomy but was decreased following thyroid or thyroxine administration. There are several exceptions, however. Grumbrecht (1939) reported an increase rather than a decrease in ovarian response to a gonadotrophin following thyroid feeding. Insofar as the response to pregnant mare's serum in the rat is concerned, Leonard and Hansen (1936) and Bischoff and Clarke (1941) noted that thyroidectomy had no effect while Smelser and Levin (1941) observed an increase in response.

Fluhmann (1934), Leonard and Hansen (1936) and Leonard (1936) stated that gonadotrophins of pituitary and non-pituitary origin may not be affected similarly by the same thyroid alterations. This would account for their finding an augmented effectiveness of sheep pituitary gonadotrophin following thyroidectomy, in contrast to an unaltered effectiveness of human pregnancy blood extract, human pregnancy urine and pregnant mare's serum. The later reports by Bischoff and Clarke (1941), Smelser and Levin (1941) and our observations do not bear out this supposition.

It is worthy of note that all workers who have dealt with the effects of altered thyroid status on the response to administered gonadotrophins have used only the rat. Conclusions have been drawn from these studies on the rat which may not be applicable to other species. In the reports on the effects of induced hyperthyroidism by feeding

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TABLE 1. EFFECTS OF EXPERIMENTALLY INDUCED HYPERTHYROIDISM AND HYPOTHYROIDISM ON RESPONSE TO GONADOTROPHIC AND STEROID HORMONES AS REPORTED BY DIFFERENT INVESTIGATORS

| Investigator | Date | Animal | Thyroid Status | Type of Hormone Injected | Was Latter More (+) or Less (−) Effective |
|---|---|---|---|---|---|
| Schockaert | 1931 | Immature ♂ rat | Thyroidectomized | Beef pit. extract | + |
| Van Horn | 1933 | Mature ♀ castrate rat | Fed dessicated thyroid | Estrogen | − |
| Fluhmann | 1934 | Immature ♀ rat | Thyroidectomized | Sheep pit. gonadotrophin | + |
| | | Immature ♀ rat | Thyroidectomized | Human preg. blood extract | Neither |
| | | Immature ♀ rat | Fed dessicated thyroid | {Sheep pit. gonadotrophin or Human preg. blood extract | − |
| | | Immature ♀ rat | Injected thyroxine | | − |
| Leonard and Hansen | 1936 | Immature ♂ rat | Thyroidectomized | Pregnant mare serum | Neither |
| | | Immature ♂ rat | Thyroidectomized | Human preg. urine | Neither |
| Leonard | 1936 | Immature ♀ rat | Thyroidectomized | Urine of castrate women | + |
| | | Immature ♀ rat | Thyroidectomized | Urine of man with testis tumor | + |
| Smelser | 1939 | Immature ♂ rat | Injected thyroxine | Autuitrin S | − |
| | | Immature ♂ rat | Injected thyroxine | Sheep pit. gonadotrophin | − |
| | | Castrate ♂ immature rat | Injected thyroxine | Testis extract | − |
| Grumbrecht | 1939 | Immature ♀ rat | Fed dessicated thyroxine | "Gonadotrophic substance" | + |
| Bischoff and Clarke | 1941 | Immature ♀ rat | Thyroidectomized | Pregnant mare serum | Neither |
| Smelser and Levin | 1941 | Immature ♀ rat | Thyroidectomized | Sheep pit. gonadotrophin | + |
| | | Immature ♀ rat | Thyroidectomized | Pregnant mare serum | + |
| | | Immature ♀ rat | Thyroidectomized | Post-menopausal gonadotrophin | + |
| Williams, Weinglass, Bissell and Peters | 1944 | Immature ♀ rat | Fed thiouracil | Chorionic gonadotrophin | Neither |
| Wright and Gustavson | 1947 | Castrate ♀ rat | Injected thyroxine | Estrone | − |
| | | Castrate ♀ rat | Thyroidectomized | Estrone | + |
| | | Castrate ♀ rat | Fed thiourea | Estrone | + |
| Masson | 1947 | Castrate mature ♂ mouse | Injected thyroxine | Testosterone | + |

desiccated thyroid or thyroxine, it appears doubtful that dosage levels were always adequately considered. A high dosage of thyroid hormone may have an entirely different effect on gonadotrophic activity than a low dosage, a fact which will be brought out particularly in our mouse experiment.

The objectives of the experiments reported here were (1) further to clarify the influences of induced hypothyroidism and hyperthyroidism on the response to the administration of a gonadotrophic hormone, and (2) to determine whether conclusions previously drawn from studies in the rat are also applicable to the mouse.

## PROCEDURE

A total of 110 immature male albino rats of the Michigan State College strain and 120 immature male albino mice (Rockland strain) were used for these studies. Various degrees of hyperthyroidism were induced by incorporating thyroprotein[3] in the ration (Purina Laboratory Chow) in concentrations of 0.02 to 0.64 per cent, and feeding each mixture for a ten day period. Hypothyroidism was induced by including 0.1 per cent thiouracil in the ration and feeding for four to twenty day periods. Those groups of rats and mice which were to receive thiouracil for the longer periods were started at an earlier age in order to reach approximately the same body weights at the end of the experiment as the groups treated for shorter periods. The initial weights and ages of the rats averaged 39.6 to 66.4 grams and 21 to 30 days respectively, and their final weights and age were 90.8 to 111.0 grams and about 40 days respectively. The initial weights of the mice averaged 11.5 to 14.3 grams, and their final weights averaged 17.1 to 23.4 grams.

A constant dose of pregnant mare's serum (Gonadogen)[4] one-half Cartland-Nelson unit, was always injected during the last four days of thyroprotein or thiouracil feeding. Preliminary trials with several dosages of Gonadogen indicated that one-half unit produced about a hundred per cent increase in the combined weights of the seminal vesicles and coagulating glands[5] of either rats or mice. The weight increases were calculated on a 100 gram body weight basis, and the standard deviation of the mean was determined for each group. Moore (1939), Leonard and Hansen (1936), Bishop and Leathem (1946) and others have demonstrated that testis weight in immature male rats and mice is but little affected by gonadotrophin injections, whereas a marked response occurs in accessory sex glands such as the seminal vesicles and coagulating glands.

Two control groups, one untreated and the other injected with Gonadogen only, were run with each series of thyroprotein or thiouracil treated animals. The effects of thyroprotein or thiouracil alone were also determined in both rats and mice. The experiments were conducted between April and August, 1948, during which time the animals were housed in a temperature controlled room (Mean of 74°F).

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[3] An iodinated casein product ("Protamone") containing approximately three per cent thyroxine as determined by chemical assay. "Protamone" was made available through the courtesy of the Cerophyl Laboratories Inc., Kansas City, Mo.

[4] Kindly supplied by the Upjohn Company, Kalamazoo, Mich.

[5] Henceforth referred to only as seminal vesicles.

TABLE 2. EFFECTS OF THIOURACIL OR THYROPROTEIN ON COMBINED WEIGHT OF SEMINAL VESICLES AND COAGULATING GLANDS OF IMMATURE MALE RATS AND MICE

| Species | No. per group | Treatment | Duration days | Initial wt., g. | Final wt., g. | Testis wt., mg. | Testis wt. 100 g. final body wt., mg. | Sem.* ves. wt. mg. | Sem. ves. wt. 100 g. final body wt., mg. |
|---|---|---|---|---|---|---|---|---|---|
| Rat | 5 | None | 10 | 53.0 | 103.7 | 724.6 | 698.7 | 25.1 | 24.4 |
| Rat | 5 | 0.1 % thiouracil | 10 | 63.6 | 101.4 | 861.0 | 840.8 | 22.8 | 22.4 |
| Rat | 5 | 0.32% thyroprotein | 10 | 59.6 | 110.3 | 992.8 | 900.1 | 26.1 | 23.7 |
| Mouse | 9 | None | 10 | 12.0 | 19.3 | 105.1 | 547.6 | 27.6 | 142.7 |
| Mouse | 7 | 0.1 % thiouracil | 10 | 12.6 | 20.1 | 110.4 | 549.2 | 29.4 | 146.2 |
| Mouse | 7 | 0.32% thyroprotein | 10 | 11.8 | 22.5 | 124.8 | 554.6 | 34.0 | 151.1 |

* Represents combined wt. of seminal vesicles and coagulating glands.

## RESULTS

The effects of ten days treatment with 0.1 per cent thiouracil or 0.32 per cent thyroprotein alone on the seminal vesicles of the immature rats and mice are shown on Table 2. It can be seen that neither of these substances had any noteworthy effect on the weight of the seminal vesicles. Apparently the secretion of pituitary gonadotrophins is but little affected by altering thyroid function during this stage of life in the animals. The short treatment as well as the dosages of the two substances administered may also account in part for the negative effects.

## RAT EXPERIMENTS

The effects of thiouracil on the response to Gonadogen in the rats are tabulated in Table 3. Gonadogen alone elicited a 107 per cent increase in seminal vesicle weight. Four days of feeding with thiouracil caused a slight but insignificant increase in response to Gonadogen. Ten days of thiouracil feeding almost doubled the response of the

TABLE 3. EFFECTS OF THIOURACIL ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE RATS

| No. rats | Treatment | Initial wt., g. | Final wt., g. | Testis wt., mg. | Testis wt. 100 g. final body wt., mg. | Sem.* ves. wt., mg. | Sem. ves. wt. 100 g. final body wt., mg. | Increase over controls 100 g. body wt., % |
|---|---|---|---|---|---|---|---|---|
| 5 | Controls | 61.8 | 110.4 | 606.5 | 549.1 | 26.2 | 23.6± .72† | |
| 5 | Gonadogen only | 54.8 | 110.0 | 950.9 | 856.0 | 55.2 | 49.0± 8.2 | +107.6 |
| 5 | 0.1% thiouracil for 4 days, and Gonadogen | 66.4 | 110.6 | 872.9 | 789.2 | 61.3 | 55.4± 8.8 | +134.7 |
| 5 | 0.1% thiouracil for 10 days, and Gonadogen | 63.8 | 104.2 | 940.4 | 902.5 | 73.8 | 70.9± 8.6 | +200.4 |
| 4 | 0.1% thiouracil for 10 days, and Gonadogen | 60.0 | 103.8 | 1192.8 | 1149.1 | 77.4 | 74.5± 6.7 | +211.4 |
| 5 | 0.1% thiouracil for 15 days, and Gonadogen | 40.0 | 90.8 | 1096.9 | 1208.1 | 95.3 | 104.6± 6.6 | +343.2 |
| 5 | 0.1% thiouracil for 20 days, and Gonadogen | 39.6 | 91.9 | 1177.2 | 1208.7 | 106.4 | 115.8±24.2 | +390.7 |

* Represents combined wt. of seminal vesicles and coagulating glands.

† Standard error of the mean $= \sqrt{\frac{\sum d^2}{n(n-1)}}$

TABLE 4. EFFECTS OF THYROPROTEIN ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE RATS

| No. rats | Treatment | Initial wt., g. | Final wt., g. | Testis wt., mg. | Testis wt., 100 g. final body wt., mg. | Sem.* ves. wt., mg. | Sem. ves. wt., 100 g. final body wt., mg. | | Increase over controls 100 g. body wt., % |
|---|---|---|---|---|---|---|---|---|---|
| 5 | Controls | 43.6 | 92.7 | 625.1 | 674.3 | 18.0 | 19.5 ±1.5† | av. =24.3 | |
| 11 | Controls | 52.0 | 106.7 | 1070.8 | 1003.0 | 31.2 | 29.2 ±1.9 | | |
| 5 | Gonadogen only | 43.2 | 95.1 | 848.0 | 891.3 | 53.0 | 55.7 ±2.5 | av. =50.2 | +106.5 |
| 5 | Gonadogen only | 44.3 | 88.6 | 625.8 | 706.3 | 39.7 | 44.8 ±1.6 | | |
| 5 | 0.02% thyroprotein, and Gonadogen | 41.2 | 89.1 | 666.0 | 747.7 | 46.5 | 52.2 ±2.6 | | +114.5 |
| 5 | 0.04% thyroprotein, and Gonadogen | 50.6 | 105.3 | 910.8 | 865.3 | 49.9 | 47.4 ±2.3 | | + 94.7 |
| 5 | 0.08% thyroprotein, and Gonadogen | 44.3 | 88.1 | 607.8 | 689.9 | 29.6 | 33.5 ±3.1 | | + 46.0 |
| 5 | 0.16% thyroprotein, and Gonadogen | 50.0 | 97.0 | 737.2 | 760.3 | 30.6 | 31.6 ±2.9 | | + 29.8 |
| 5 | 0.32% thyroprotein, and Gonadogen | 58.8 | 109.1 | 1036.0 | 949.6 | 40.8 | 37.3 ±6.6 | av. =36.0 | + 47.8 |
| 5 | 0.32% thyroprotein, and Gonadogen | 45.6 | 88.8 | 704.5 | 793.1 | 30.9 | 34.8 ±2.5 | | |
| 5 | 0.64% thyroprotein, and Gonadogen | 51.0 | 106.5 | 821.1 | 770.8 | 30.1 | 28.3 ±2.9 | | + 16.0 |

* Represents combined wt. of seminal vesicles and coagulating glands.
† Standard error of the mean.

seminal vesicles to Gonadogen injection. The fifteen and twenty day treatments with thiouracil at least tripled the response to Gonadogen. It is evident that as the degree of thiouracil-induced hypothyroidism was increased in the rat, the greater was the response to a constant dose of pregnant mare's serum.

Thyroprotein feeding reduced the effectiveness of Gonadogen (Table 4). Gonadogen alone elicited a 107 per cent increase in weight of the seminal vesicles. The addition of 0.02 or 0.04 per cent thyroprotein produced no significant alteration in gonadotrophic response, but higher concentrations progressively inhibited the response. The highest level of thyroprotein fed, 0.64 per cent, appears to have completely inhibited the action of the pregnant mare's serum on the seminal vesicles.

TABLE 5. EFFECTS OF THIOURACIL ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE MICE

| No. mice | Treatment | Initial wt., g. | Final wt., g. | Testis wt., mg. | Testis wt., 100 g. final body wt., mg. | Sem.* ves. wt., mg. | Sem. ves. wt., 100 g. final mg. | Increase over controls 100 g. body wt., % |
|---|---|---|---|---|---|---|---|---|
| 8 | Controls | 12.5 | 22.8 | 158.2 | 693.3 | 36.2 | 158.5±14.7† | |
| 6 | Gonadogen only | 13.3 | 17.3 | 111.9 | 648.3 | 55.2 | 321.7± 4.9 | +102.9 |
| 8 | 0.1% thiouracil for 4 days, and Gonadogen | 13.7 | 17.1 | 113.4 | 661.3 | 38.2 | 222.9±17.4 | + 40.6 |
| 8 | 0.1% thiouracil for 7 days, and Gonadogen | 13.2 | 21.1 | 125.0 | 592.8 | 42.7 | 202.7±21.5 | + 27.8 |
| 8 | 0.1% thiouracil for 10 days, and Gonadogen | 12.2 | 20.4 | 125.7 | 616.3 | 50.7 | 248.4±23.2 | + 56.7 |
| 8 | 0.1% thiouracil for 15 days, and Gonadogen | 12.0 | 21.8 | 143.0 | 657.0 | 51.4 | 236.1±14.2 | + 48.9 |
| 6 | 0.1% thiouracil for 20 days, and Gonadogen | 11.5 | 20.9 | 154.7 | 139.6 | 53.6 | 256.5±22.3 | + 61.8 |

* Represents combined wt. of seminal vesicles and coagulating glands.
† Standard error of the mean.

## MOUSE EXPERIMENTS

In contrast to the rat, the effectiveness of Gonadogen in the mouse was reduced by thiouracil and augmented by thyroprotein feeding. In the thiouracil series (Table 5), the pregnant mare's serum alone caused a 103 per cent increase in seminal vesicle weight. The thiouracil treated groups all showed a significantly reduced capacity to respond to Gonadogen, although in no group was the response completely inhibited.

The feeding of thyroprotein caused an increase in response to Gonadogen in all groups, with the exception of the group fed the highest concentration of thyroprotein, 0.64 per cent (Table 6). The latter level of thyroprotein slightly reduced the effectiveness of Gonadogen, emphasizing the importance of dosage levels of thyroidal substances. In general, it can be stated that immature male mice and immature male rats react oppositely to the same gonadotrophic stimulus during similar alterations in thyroid function.

TABLE 6. EFFECTS OF THYROPROTEIN ON RESPONSE TO A CONSTANT DOSE OF GONADOGEN IN IMMATURE MALE MICE

| No. mice | Treatment | Initial wt., g. | Final wt., g. | Testis wt., mg. | Testis wt., 100 g. body final wt., mg. | Sem.* ves. wt., mg. | Sem. ves. wt., 100 g. final body wt., mg. | Increase over controls 100 g. body wt., % |
|---|---|---|---|---|---|---|---|---|
| 9 | Controls | 12.0 | 19.4 | 105.1 | 547.6 | 27.6 | 142.7±10.6† | |
| 9 | Gonadogen only | 11.6 | 20.3 | 116.4 | 572.6 | 46.1 | 226.9±19.5 | + 58.9 |
| 6 | 0.02% thyroprotein, and Gonadogen | 12.0 | 22.1 | 133.3 | 602.7 | 76.1 | 344.5±12.7 | +141.4 |
| 6 | 0.04% thyroprotein, and Gonadogen | 13.6 | 23.3 | 140.7 | 603.7 | 77.8 | 333.9±17.1 | +134.0 |
| 6 | 0.08% thyroprotein, and Gonadogen | 14.3 | 23.4 | 129.3 | 551.8 | 74.6 | 318.5±31.8 | +123.2 |
| 5 | 0.16% thyroprotein, and Gonadogen | 14.0 | 22.1 | 141.1 | 637.9 | 72.6 | 328.0±18.9 | +129.9 |
| 5 | 0.32% thyroprotein, and Gonadogen | 14.2 | 22.4 | 145.0 | 647.7 | 64.5 | 288.2±24.6 | +101.9 |
| 8 | 0.64% thyroprotein, and Gonadogen | 12.7 | 21.2 | 134.0 | 630.9 | 41.6 | 195.9±11.7 | + 37.2 |

* Represents combined wt. of seminal vesicles and coagulating glands.
† Standard error of the mean.

## DISCUSSION

It cannot be maintained that the normal immature male rat is "hyperthyroid" or that the normal immature male mouse is "hypothyroid," since such terms are more properly reserved for individuals with particular syndromes rather than for an entire species. The data presented here indicate that, insofar as the response to pregnant mare's serum is concerned, immature male rats are secreting more than an optimal amount of thyroid hormone, whereas immature male mice are secreting less than an optimal amount (Fig. 1). Studies of the thyroid secretion rates of rats (Monroe and Turner, 1946) and mice (Hurst and Turner, 1948) do not reveal any marked differences between the two species. However, other lines of evidence substantiate the idea that the immature rat is secreting more than an optimal amount of thyroid hormone for certain processes when compared to the immature mouse.

Astwood (1945) demonstrated that the administration of 0.01 per cent thiouracil in the feed of rats for a period of 9.5 months beginning at weaning time increased the growth rate over controls. This increase was reflected in a proportional gain in skeletal dimensions. In mice, Hurst and Turner (1948) reported that thiouracil fed at several levels invariably retarded growth, and this was reflected in a greater fat content of the carcasses. The administration of thyroxine or thyroprotein by Koger and Turner (1943) was found to retard growth in rats but to increase growth in mice. The increased body weight of the mice was reflected in a greater retention of protein and water and a reduction in fat content. A recent report by Fogelman and Ivy (1948) indicates that rats are secreting more than an optimal amount of thyroid hormone for another growth process. They noted that administration of thiouracil to partially hepatectomized rats increased the rate of liver regeneration when compared with controls.

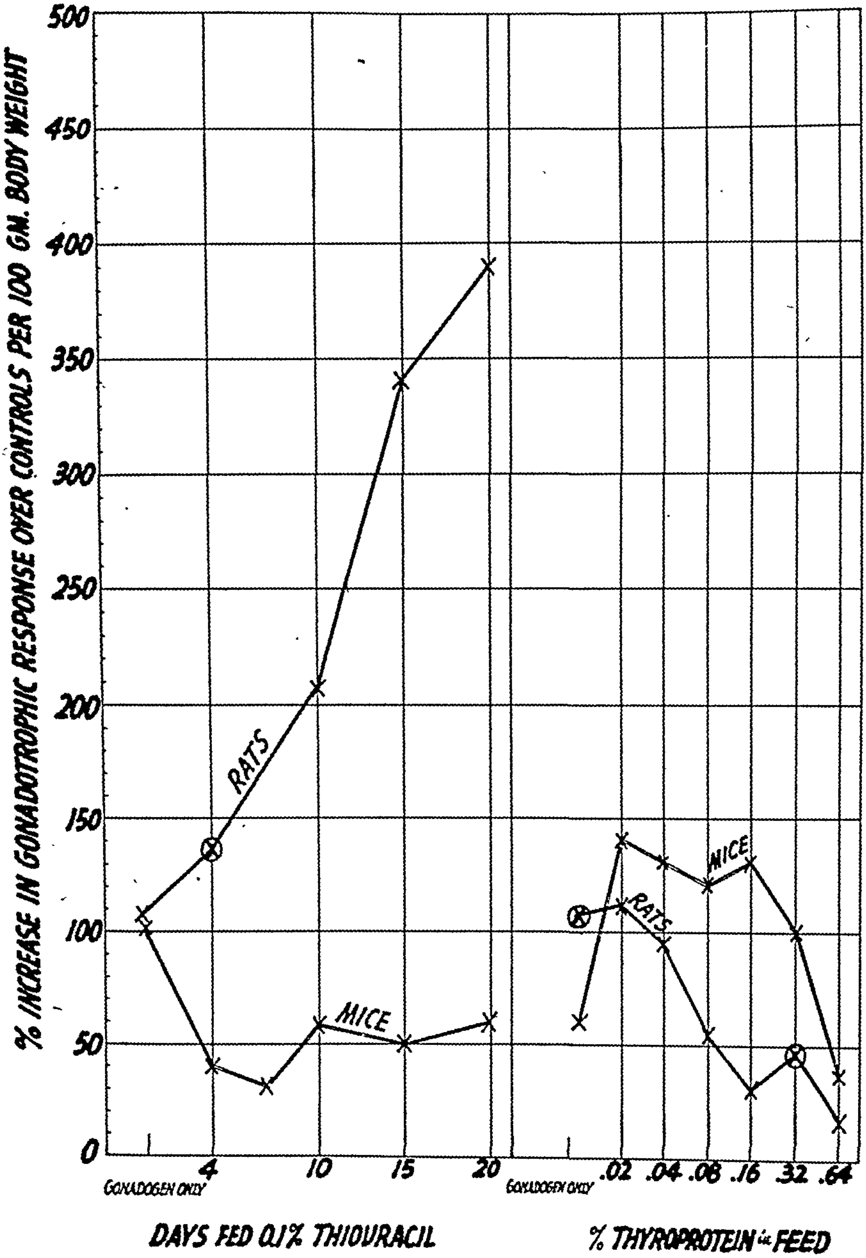


FIG. 1. The percent increase in the combined weights of the seminal vesicles and coagulating glands following treatment with Gonadogen only, and with Gonadogen plus thiouracil or thyroprotein.

⊗ Average of two groups.

Recent work on mammary growth in rats and mice further illustrates the dissimilarities in the effects of experimentally altered thyroid function in these species, Leonard and Reece (1941), Smithcors and Leonard (1943), Trentin, Hurst and Turner (1948) and Johnston and Smithcors (1948) found that thyroidectomy or thiouracil administration augmented mammary growth in the rat. However, similar treatment in the mouse was found to decrease mammary growth by Mixner and Turner (1943), Mixner (1947) and Trentin, Hurst and Turner (1948). Mixner and Turner (1943) also reported that the administration of thyroxine to ovariectomized mice increased the mammary growth response to injections of estrogen and progesterone.

All of the above findings appear to support the conclusion that growing rats secrete more than an optimal amount of thyroid hormone while growing mice secrete less than an optimal amount insofar as the reactivity of certain body tissues and organs are concerned. A different theory, however, was offered by Bischoff, Clarke and Epps (1941) to explain the effects of thyroidectomy or thyroid administration on the response to gonadotrophins in rats. They believed the altered thyroid status either decreased or increased the exchange of body fluids, thus decreasing or increasing the rate of hormone resorption. The observations in the mouse, however, appear to make such a conclusion untenable. Obviously it is illogical to suppose that thyroid administration increases the rate of exchange of body fluids in the rat but decreases it in the mouse.

It is believed the data presented in this paper, as well as related work of other investigators, definitely establishes the existence of an important species difference between young rats and mice. It can be stated that young rats secrete more than an optimal amount of thyroid hormone while young mice secrete less than an optimal amount of thyroid hormone for a variety of body functions and reactions. This suggests that other species, and perhaps some strains and individuals within a species, also may not be secreting optimal amounts of thyroid hormone for certain body functions. There is the possibility, therefore, of inducing degrees of hypothyroidism or hyperthyroidism which would insure maximum responsiveness to certain stimuli. These possibilities are already being explored in the field of animal research, where thiouracil and thyroprotein are being fed at various levels to farm animals in an attempt to increase productive processes such as growth, fattening, milk yield and egg production.

## SUMMARY

The effects of experimentally induced hyper- or hypothyroidism on the response to a constant dose of pregnant mare's serum ("Gonadogen") were determined in immature male rats and mice. Hypothyroidism was produced by feeding 0.1 per cent thiouracil for four to twenty day periods and hyperthyroidism was produced by feeding

thyroprotein in concentrations of 0.02 to 0.64 per cent for ten day periods. The increase in the combined weight of the seminal vesicles and coagulating glands was used to measure the response to the pregnant mare's serum.

Thiouracil or thyroprotein, when fed alone, had no effect on the weight of the seminal vesicles and coagulating glands. When pregnant mare's serum was injected, the response of the seminal vesicles and coagulating glands was partially to completely inhibited by all except the lowest levels of thyroprotein in the rat, while in the mouse all except the highest concentration of thyroprotein increased the gonadotrophic response by 72 to 140 per cent. Thiouracil, particularly when fed for the longer periods, increased the gonadotrophic response in rats by as much as 300 per cent, while the response in mice was reduced by 40 to 73 per cent.

It is concluded that, insofar as the response to pregnant mare's serum is concerned, immature male rats are secreting more than an optimal amount of thyroid hormone whereas immature mice are secreting less than an optimal amount of thyroid hormone. A discussion of these and related data by other investigators leads to the conclusion that there is a distinct species thyroid difference between the rat and mouse which accounts for their opposite reactions to a number of similar stimuli.

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# THE QUANTITATIVE RELATION BETWEEN CERTAIN AMINO ACIDS AND GLYCOGENESIS AS INFLUENCED BY ADRENALECTOMY AND ADRENAL REPLACEMENT[1]

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## INTRODUCTION

THE effect of adrenal cortical function upon carbohydrate and protein metabolism has been repeatedly demonstrated. Much evidence has accumulated to indicate that there is a direct effect of certain cortical principles upon the rate of gluconeogenesis from protein, but there is little knowledge as to the reactions involved. Several possible mechanisms have been postulated. Long, Katzin and Fry (1940) suggest that the cortical principle accelerates the mobilization of endogenous proteins. Wells and Kendall (1940) have reported that the phlorhizinized, adrenalectomized rat forms glucose from fed protein at a normal rate. Further evidence in favor of this mechanism is offered by Evans (1941), who found that the rate of deamination of alanine proceeded at a normal rate in the adrenalectomized rat.

The possibility that the cortical hormones exert their effect upon the rate of deamination of amino acids was investigated by Lewis, et al (1940). They observed that the formation of glucose from alanine in the adrenalectomized, phlorhizinized rat was reduced. Russell and Wilhelmi (1941) found that kidney slices from adrenalectomized rats produce less than normal amounts of carbohydrate from alanine and glutamic acid. Jimenez—Diaz (1936) reported a subnormal rate of deamination in the kidneys of adrenalectomized cats. Samuels, et al (1937) found that cortical insufficiency in the rat caused a decrease in the formation of liver glycogen from fed alanine. These and other prevailing theories are discussed at length by Long (1941).

A new approach to this problem was offered by the development of quantitative paper chromatography (Awapara, 1949) for the estimation of some free amino acids in tissues. It appeared important to extend, by the use of this technique, the study of the role of the

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adrenal glands in the conversion of protein to glycogen. The results of a study of liver glycogen and alanine, glycine, glutamic acid and aspartic acid in hepatic and muscle tissue of normal rats, untreated adrenalectomized rats, and adrenalectomized rats given substitution therapy are herewith presented. The replacement materials consisted of 17-hydroxydehydrocorticosterone and desoxycorticosterone acetate, referred to subsequently as Compound E (Kendall) and DOCA, respectively.

## EXPERIMENTAL

Male rats, of the Sprague-Dawley strain, weighing 130–145 gm., were used in this study. The adrenal glands were removed under light ether anesthesia through separate incisions. Care was exercised to remove as much of the periadrenal adipose tissue as possible, and each adrenal gland was inspected after excision for gross evidence of incomplete removal. The body wall was closed with gut, and the skin incision repaired with skin clips. Reasonable asepsis was observed during the procedure.

Immediately following the operation, the rats were given physiological saline and ordinary food *ad libitum*. The animals were weighed at the time of the operation, and twice during the subsequent six day period. Only those animals surviving and showing a weight gain during this period were used.

The adrenalectomized rats receiving either Compound E or DOCA were given distilled water for drinking purposes during the 24 hour fasting period preceding autopsy. The third group of adrenalectomized rats received no treatment other than physiological saline during the fasting period. Three mg. of Compound E and 1.0 mg. of DOCA in 1.5 ml. and 0.6 ml. of corn oil, respectively, were given each rat of the appropriate groups. The total dose was given in three, equally divided, subcutaneous injections during the first 8 hours of the 24 hour fast.

At the time of autopsy, the rats were killed with preliminary anesthesia by a lethal, intraperitoneal injection of nembutal. A sample of liver weighing approximately 1 gm. was quickly removed and placed in hot 30 per cent potassium hydroxide for hepatic glycogen determinations. A second liver sample was taken for the amino acid determinations. The remainder of the liver of each alternate rat was dried for 24 hours at 100°C. in order to determine the percentage of total solids. A sample of the gastrocnemius muscle of each rat was also removed for determination of amino acids.

Each rat was carefully examined for residual adrenal tissue macroscopically and none of the operated rats was found to have such. The rats receiving Compound E and the controls lost practically twice as much weight during the 24 hour fast, as did the rats receiving desoxycorticosterone acetate or only salt water during the same period.

Liver glycogen was determined by the method of Good, Kramer and Somogyi (1933). Amino acids were extracted from tissue by the method of Awapara (1948) and measured by the technique of quantitative paper chromotography (Awapara, 1949).

## RESULTS AND DISCUSSION

In Table 1 are shown the results of analyses for glycogen in liver and for free amino acids in liver and muscle of the experimental and

normal control animals. The increase of glycogen in the livers of Compound E-treated animals over that of normal rats, adrenalectomized rats maintained on sodium chloride, or rats treated with DOCA, is confirmatory of many previous observations. There is a striking decrease in the concentration of free dicarboxylic amino acids in the liver following administration of Compound E. This is in contrast to the results obtained by desoxycorticosterone acetate or saline treatment. The diminution in concentration of these amino acids as compared

TABLE ONE

| STATUS — GIVEN | LIVER GLYCOGEN PER CENT | LIVER: MICROGRAMS OF AMINO NITROGEN PER GRAM FRESH TISSUE | | | | MUSCLE: MICROGRAMS OF AMINO NITROGEN PER GRAM FRESH TISSUE | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | SUM | DI-COOH ACID FRACTION | GLYCINE | ALANINE | SUM | DI-COOH ACID FRACTION | GLYCINE | ALANINE |
| INTACT — NONE | 0.95 ±0.17* | 313 ±18.13 | 119 ±5.10 | 107 ±12.80 | 87 ±9.01 | 428 ±39.99 | 58 ±2.70 | 279 ±34.17 | 91 ±12.11 |
| ADRENAL — COMP. E | 1.50 ±0.38 | 211 ±13.11 | 78 ±4.22 | 83 ±8.37 | 50 ±3.37 | 315 ±11.88 | 58 ±9.21 | 203 ±11.04 | 55 ±4.64 |
| ADRENAL — D.O.C.A. | 0.15 ±0.02 | 255 ±19.09 | 140 ±18.78 | 67 ±11.52 | 49 ±5.00 | 298 ±18.84 | 38 ±3.49 | 202 ±18.11 | 59 ±2.51 |
| ADRENAL — SALINE | 0.19 ±0.05 | 321 ±26.65 | 167 ±13.30 | 93 ±11.70 | 61 ±4.12 | 293 ±14.91 | 43 ±3.30 | 180 ±11.32 | 69 ±5.27 |

*STANDARD ERROR CORRECTED FOR SMALL NUMBERS

TABLE 1. CONCENTRATION OF GLYCOGEN AND FREE AMINO ACIDS IN LIVER AND MUSCLE

with the results in normal, unoperated animals is less marked, but roughly proportional to the glycogen differences demonstrated between these two groups. The changes in amino acid concentration and distribution in muscle were statistically insignificant. This finding corresponds to the moderate changes, and often difficultly demonstrable effects of adrenalectomy and steroid hormone treatment on muscle glycogen.

In Table 2 the amino acid concentrations are recorded with reference to the total free amino acid nitrogen represented by the summation of glutamic acid, aspartic acid, alanine and glycine. The only striking observation to be made here is the relative excess of free

alanine in the livers of unoperated controls and Compound E-treated, adrenalectomized rats.

An abundance of previous work demonstrates that adrenal cortical insufficiency is associated with impaired glycogenesis from protein. Normal conversion of protein to carbohydrate is restored in the adrenalectomized animal by administration of the C-11 oxygenated corticosteroids. The 11-desoxycorticosteroids, although highly active with respect to electrolyte and water metabolism, do not stimulate

## TABLE TWO

| GROUP | $\Sigma F = 1.00$ | DI-COOH FRACTION | GLYCINE | ALANINE |
|---|---|---|---|---|
| *RATIO OF EACH FRACTION OF LIVER TO SUM OF LIVER FRACTIONS* | | | | |
| NORMAL | 1.00 ±0.083 | 0.38 ±0.024 | 0.34 ±0.046 | 0.28 ±0.033 |
| COMP. E | 1.00 ±0.087 | 0.37 ±0.031 | 0.39 ±0.046 | 0.24 ±0.022 |
| D.O.C.A. | 1.00 ±0.106 | 0.55 ±0.084 | 0.26 ±0.049 | 0.19 ±0.024 |
| SALINE | 1.00 ±0.116 | 0.52 ±0.059 | 0.29 ±0.044 | 0.19 ±0.020 |
| *RATIO OF EACH FRACTION OF MUSCLE TO SUM OF MUSCLE FRACTIONS* | | | | |
| NORMAL | 1.00 ±0.127 | 0.14 ±0.015 | 0.65 ±0.099 | 0.21 ±0.034 |
| COMP. E | 1.00 ±0.053 | 0.18 ±0.029 | 0.64 ±0.042 | 0.18 ±0.017 |
| D.O.C.A. | 1.00 ±0.089 | 0.13 ±0.014 | 0.68 ±0.074 | 0.20 ±0.015 |
| SALINE | 1.00 ±0.072 | 0.15 ±0.015 | 0.61 ±0.050 | 0.24 ±0.022 |

S.E. OF QUOTIENT $= Q\sqrt{(S.E._1/M_1)^2 + (S.E._2/M_2)^2}$

TABLE 2. RATIOS OF INDIVIDUAL AMINO ACIDS TO TOTAL AMINO ACIDS DETERMINED

the formation of glycogen from non-carbohydrate sources. Experimental evidence presently available does not provide unequivocal support for any theory attempting to explain the mechanism, nor does it identify the amino acids through which the 11-oxycorticosteroids stimulate glycogenesis. However, the well established position of the dicarboxylic amino acids and alanine as intermediates in the transformation of protein to carbohydrate through the aminopherase (transamination) mechanism suggests a possible explanation for the data recorded in Table 1. The action of Compound E in promoting glycogen synthesis was accompanied by a diminished concentration of the free dicarboxylic amino acids of liver tissue. This finding may

reasonably be interpreted as indicating the transformation of these amino acids to carbohydrate. This conclusion is supported further by the data recorded in Table 2. Here we note that glycogen synthesis is accompanied by a relative increase in the proportion of alanine to the dicarboxylic amino acids. This finding is explicable in terms of the aminopherase mechanism, since the reaction rates of both glutamic acid and aspartic acid with pyruvic acid in liver favor the production of alanine. Our figures for amino acid distribution in muscle may be correlated with the fact that the aminopherase reactions proceed at low, inappreciable rates in that tissue.

Although these findings suggest that carbohydrate synthesis stimulated by Compound E takes place through the aminopherase mechanism and involves the utilization of the dicarboxylic amino acids, more complete studies will be necessary to establish this fact. Cohen has pointed out that in the field of intermediary metabolism, final identification of a process requires not only a determination of change in the reacting substances, but also a demonstration of the enzyme mechanism concerned.

The absorption, transport and deposition of carbohydrate in the adrenalectomized animal is known to approach the normal so long as maintenance amounts of sodium chloride are fed or there is adequate treatment with the 11-desoxycorticosteroids. If, however, animals so treated are subjected to fasting or any other conditions requiring the carbohydrate stores to be replenished from protein sources, the C-11 oxygenated corticosteroids must be available if an effective adaptation is to be accomplished. The conditions of our experiment, imposing only a 24 hour fast, are not calculated to show the maximum deficiency in carbohydrate metabolism in the animals deprived of the 11-oxysteroids. No doubt, the trends exhibited here could be considerably magnified by applying more strenuous conditions.

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### SUMMARY

The increase in liver glycogen of Compound E-treated, adrenalectomized rats over normal rats, or adrenalectomized rats given salt or desoxycorticosterone acetate is reaffirmed. The dicarboxylic amino acids are decreased and alanine is increased in the livers of adrenalectomized rats given Compound E. The changes in amino acid concentration and distribution in muscle were not impressive. The relation of these findings to current theories is discussed.

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# THE REACTION OF MALE FROGS TO EPINEPHRINE[1]

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WITH the exception of a few widely scattered reports (Galli-Mainini, 1947a, 1947b, 1947c; Houssay, 1947), almost all the information available today on the hormonal reactivity of male frogs and toads pertains to the effect of gonadotropins on these amphibia (Robbins, Parker and Bianco, 1947; Galli-Mainini, 1947a, 1948; Wiltberger and Miller, 1948; Miller and Wiltberger, 1948; Robbins and Parker, (literature) 1948). It has been already shown that a number of species of these male amphibia are available for endocrine assay work. The two male species most widely used at present for pregnancy diagnosis work are the South American toad, Bufo arenarum, and the North American spotted frog, the Rana pipiens. In pregnancy diagnosis, reports to date indicate that both animals appear to be quite satisfactory, giving virtually no false positives and very few false negatives (Galli-Mainini, 1948; Robbins and Parker, 1948). In contrast to the above two species the male Xenopus laevis, a South African frog, while a sensitive assay animal for gonadotropin of pituitary origin, has proved to be of little value in pregnancy diagnosis. While it reacts to gonadotropin of chorionic origin and thus faithfully detects pregnancy, it at the same time, however, gives many false positive reactions in women known to be not pregnant (unpublished work). Repeated efforts to isolate the substance or substances in urine producing these false positive reactions have been completely unsuccessful. No correlations have been drawn between these false positives and the diet of the patient, the ingestion of drugs or the phase of the menstrual cycle. It is of interest that these reactions were encountered only in girls having regular normal menses, not in patients with amenorrhea. A nonspecific alarm reaction to noxious agents contained in the urine with the release of adrenal hormones was considered as the possible basis of these false positive reactions. Accordingly, adrenocorticotropic hormone, desoxycorticosterone acetate, dehydro-isoandrosterone and epinephrine were administered subcutaneously to male Xenopus laevis and a

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rapid, strongly positive reaction to the epinephrine ensued. The corticotropic hormone, as well as the steroids, evoked no response. This reaction to epinephrine, while of great interest, seemed unlikely to be the rational explanation of the false positive tests encountered in the Xenopus laevis, since, to the best of our knowledge, epinephrine has never been identified in urine. However, the speed of the reaction and the simplicity of demonstrating this response suggested the possible applicability of this technic for the bio-assay of epinephrine preparations. Studies, therefore, on the reactivity of the male Rana pipiens and Xenopus laevis to epinephrine, as well as to various other autonomic drugs, form the basis of this present report.

## EXPERIMENTAL DATA

Throughout these assay experiments adult male frogs procured from commercial collectors were used. The specimens of Xenopus laevis, had been gathered from their native habitat, South Africa, and weighed between 35 and 55 grams. The Rana pipiens species of frog were of domestic origin, weighing approximately 35 to 45 grams, and were for the most part kept in artificial hibernation throughout the year after being collected during the summer months.

These studies carried on throughout a year found the Xenopus laevis satisfactory for adrenalin assay at all times; the Rana pipiens, on the contrary, appeared to pass through a phase of adrenalin nonreactivity during the months of June, July and August. The basis of this transient refractoriness is totally obscure. All the preparations were used in aqueous form and were administered subcutaneously to the amphibia into the dorsal lymph sac. Reactions to these drugs, the emission of sperm from the cloacal tract, were followed by microscopic examination of the cloacal fluid, using the technic previously described (Robbins, Parker and Bianco, 1947).

Epinephrine of natural origin (Parke Davis, 1–1000 and Lederle, 1–1000) was administered to male amphibia, Rana pipiens, Xenopus laevis and Bufo arenarum, in doses of 0.1 cc. (0.1 mg.). The first two species reacted within one hour, the Bufo arenarum remaining negative indefinitely. No other effects of this drug on the animals were noted. To exclude the possibility that this positive reaction was caused by some side effect of contained animal substances present in the naturally derived epinephrine, synthetic hormone (Burroughs-Wellcome, 1–100) was likewise employed. Doses of 0.1 mg. of this synthetic preparation were equally effective in producing a positive response in the Rana pipiens and Xenopus laevis.

By the use of serial dilution technics it could be shown that both species of frogs, Xenopus laevis and Rana pipiens, would react to extremely small doses of epinephrine varying with individual animals between 0.01 mg. and 0.001 mg. Moreover, the reaction to this amount of drug remained remarkably constant for large numbers of adult males of both species, despite variations in the body weight of individual animals.

In an effort to determine whether this reaction to epinephrine was essentially the effect of the Sympathin "E" or "I" content, commercial preparations of these fractions were given to both types of frogs. The source of Sympathin I was Isuprel, 1–200 (Winthrop-Stearns); Arterenol, 0.1 mg., was used as a source of the "E" fraction. Both substances produced positive results in the doses indicated in the table which follows. A wide variety of other adrenergic drugs was administered to these amphibia with the results indicated in Table I.

TABLE 1

| Substance | Xenopus laevis | | Rana pipiens | |
|---|---|---|---|---|
| | Dose | Reaction[1] | Dose | Reaction[1] |
| Epinephrine (Parke Davis, 1–1000) | 0.01 mgm. | ++++ | 0.01 mgm. | ++++ |
| Epinephrine (Lederle, 1–1000) | 0.001 mgm. | ++++ | 0.003 mgm. | ++++ |
| Epinephrine (synthetic Burroughs-Wellcome, 1–100) | 0.001 mgm. | ++++ | 0.001 mgm. | ++++ |
| Isuprel (Sympathin I) | 0.1 mgm. | ++++ | 0.1 mgm. | ++++ |
| L-Arterenol (Sympathin E) | | | 0.4 mgm. | ++++ |
| Neosynephrine | 5.0 mgm. | 0 | 25.0 mgm. | +++ |
| Benzedrine sulfate | 1.0 mgm. | ++++ | 1.0 mgm. | 0 |
| Ephedrine sulfate | 5.0 mgm. | ++ | 5.0 mgm. | 0 |
| Mecholyl chloride | 5.0 mgm. | 0 | 5.0 mgm. | 0 |
| Eserine sulfate | 0.64 mgm. | 0 | 0.64 mgm. | 0 |
| Pilocarpine | 4.0 mgm. | 0 | 4.0 mgm. | 0 |
| Shock reaction to trauma | | 0 | | |

[1] The reactions were graded 1+ to 4+ as follows:
+ =single sperm per high power field.
++ =several sperm per high power field.
+++ =most high power fields containing sperm too numerous to count.
++++ =all high power fields containing sperm too numerous to count.

Painful stimuli as well as crush injury to the hind extremity produced no emission of sperm, and in addition the administration of Carcholin (Merck), 0.1 mg., presumably stimulating the release of endogenous adrenalin in eserinized animals, evoked no response. Larger doses of Carcholin proved toxic to these animals.

In an effort to determine whether epinephrine was producing its effect through the pituitary, hypophysectomy by the method of Hogben (1923) was performed on several animals. The hypophysectomized amphibia reacted to epinephrine equally as well as the normal controls.

## DISCUSSION

From observations on several hundreds of male Xenopus laevis and Rana pipiens it is apparent that these amphibia react to subcutaneously administered epinephrine by the emission of sperm. This response occurs rapidly, almost invariably within one hour after the administration of the drug. It is a readily demonstrable reaction as well as a remarkably constant one. The fact that graded doses down to a level of 0.01 to 0.001 of a mg. of epinephrine produce a response, doses below this level being inactive, strongly suggests some definite threshold phramacologic action of the drug. The positive reaction of Xenopus laevis to epinephrine carries over to such closely allied drugs as benzedrine and ephedrine, suggesting the possibility that some common chemical fraction or radical is the actual effective agent. It is interesting to note that some species difference exists between the Xenopus laevis and Rana pipiens in their reactivity to other ad-

renergic drugs since the latter type, Rana pipiens, fails to respond to any drugs other than epinephrine and neosynephrine. The reason for these differences is totally obscure. Both Sympathin I and Sympathin E produce positive reactions in the Rana pipiens. With the Xenopus laevis a positive reaction was obtained with Sympathin I, insufficient "E" being available for testing on this frog. None of the cholinergic drugs (acetyl Beta choline, acetyl choline or eserine) proved to be effective in producing a reaction, an observation in keeping with the apparent specificity of this response to adrenergic drugs.

The mechanism by which adrenalin and its related compounds produce emission of sperm in these frogs is extremely intriguing. At the present time, it has not yet been elucidated and work on this problem continues. Several aspects, however, seem worthy of note. The activity of so-called Sympathin I and E strongly suggests that epinephrine may be producing its effects by upsetting some finely balanced mechanism, either by the release of some block, such as some sphincteric cut-off, or by exciting some ejaculatory mechanism which overpowers the blocking mechanism. It is clear that the response of these male frogs to adrenalin does not represent simply a nonspecific reaction to bodily insult since noxious stimuli, pain and crushing injuries fail to produce the emission of sperm. The absence of a reaction to Carcholin, implying that endogenous adrenalin is ineffective in evoking the same response as exogenous adrenalin, may simply indicate failure of the effective release of the epinephrine by this drug. On the other hand, it is entirely possible that insufficient adrenal tissue is present in the frog to produce effective amounts of epinephrine. Microscopic search of the kidneys of these amphibia revealed only apparent adrenal cortical cells, never any tissue suggestive of adrenal medulla. In support of this possibility is the existence of a not inconsiderable controversy over the actual presence of a functioning adrenal gland in many amphibia. Markee et al. (1948) have shown that in the rabbit direct intrapituitary installation of adrenalin will produce the release of luteinizing gonadotropin. To rule out the possibility that these adrenergics might be thus acting on the genital system through the pituitary, hypophysectomy was performed on numerous frogs without effect on the reaction to epinephrine or benzedrine. Removal of the pituitary failed to alter the sensitivity of these amphibia to these drugs. In conclusion, therefore, at the present writing the mode of action of these epinephrine substances remains obscure.

Despite the lack of knowledge as to the "modus operandi" the empiric observation that male Rana pipiens and Xenopus laevis will respond to a relatively constant dose of epinephrine provides a most valuable tool for pharmacologic assay, providing a substitute for present laborious blood-pressure studies on dogs. Injection of a series of graded dilutions of epinephrine of unknown strength into male frogs will rapidly disclose the approximate content of the drugs in

these unknown solutions, since it can be reasonably certainly assumed from this study that the endpoint dose contains 0.01–0.001 mg. of epinephrine. Thus unknown solutions may be titered by the use of male frogs to reasonably close tolerances within 1–2 hours. The simplicity of performance of this technic, as well as its economy and ease of maintaining colonies of frogs, renders this procedure of real value.

Several limitations deserve re-emphasis. In summer months the sensitivity of Rana pipiens, derived from native habitats, to adrenalin is considerably lowered, rendering this use of these frogs during this time undesirable, a defect not encountered in the Xenopus laevis. Whether Rana pipiens kept in enforced hibernation from the previous summer will show this seasonal insensitivity to adrenalin is not known. The second point of caution is that the two species of toads, namely, Bufo arenarum Hensel and Bufo fowleri, both fail to respond to adrenalin. Galli-Mianini (1948) in a recent report confirms this observation in the Bufo arenarum. This species difference in reactivity to adrenalin is in itself a most interesting point, suggesting possible differences in the makeup of the endocrine or genital systems between various species of amphibia.

## SUMMARY

It has been shown that certain male amphibia respond to the subcutaneous administration of epinephrine by the emission of spermatozoa, the South African Xenopus laevis being reactive throughout the year, the Rana pipiens passing through a refractory period during the summer months of June, July and August. This reaction to epinephrine appears to be a remarkably constant one in which most male Xenopus laevis and Rana pipiens adults, despite some variation in body weight, always respond to dosage levels between 0.01 and 0.001 mgms. Synthetic as well as naturally derived epinephrine are equally effective and hypophysectomy in no way alters the reactivity of the animal. With the Xenopus laevis this epinephrine reactivity is carried over to the adrenergic drugs, benzedrine and ephedrine.

The possible practical application of this male amphibian reaction to the bio-assay of epinephrine preparations is discussed.

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# ASSOCIATION NOTICE

## ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

## POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

# ASSOCIATION AWARDS FOR 1949

## THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

## THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

## THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

ENDOCRINOLOGY

VOLUME 44 MAY, 1949 NUMBER 5

# ALTERED GLUCOSE TOLERANCE WITH HISTOLOGICALLY NORMAL ISLETS FOLLOWING REPEATED SMALL DOSES OF ALLOXAN[1]

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DUNN AND COWORKERS (1943) observed beta cell necrosis in the pancreatic islets of rats following the administration of alloxan subcutaneously (200–400 mg./kg.) in a single injection. Such animals became permanently diabetic if they survived the initial toxic effects of the chemical. Shipley and Rannefeld (1945) noted the decreased glucose tolerance of rats receiving 25 mg./kg. doses intravenously. Hyperglycemia and glucosuria were not, however, exhibited by these animals.[3] To correlate this altered glucose tolerance with some histological evidence, if present, of beta cell impairment was the purpose of the experiment being reported.

## EXPERIMENTAL

Five groups of albino rats of mixed sex were used. Animals weighed about 100 grams at the start of the experiment, and were weighed every 10 days while under observation. They were fed Purina Fox Chow ad libitum. The stock of rats of the Department of Anatomy colony, although not inbred by brother-sister matings, is quite uniform genetically.

Alloxan[4] in 4 per cent aqueous solution was injected intravenously every other day in doses of 20 mg./kg. of body weight. One group of rats received 2, one 4, and a third group 10 doses. A fourth group received a single dose of 30 mg./kg., and a fifth group received no al-

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[1] This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[2] Senior Research Fellow, USPHS.

[3] Single dose of 30 mg./kg. given intravenously to rats in this laboratory induced beta cell necrosis with persistent hyperglycemia and glucosuria.

[4] Alloxan monohydrate—Eastman Kodak Co.

loxan. Glucose tolerance tests were conducted 10 days after the last injection of alloxan by withdrawing a 16 hour fasting blood sample, injecting 0.5 g./kg. glucose intravenously in 20 per cent solution, and drawing blood samples at one-half, one, and 2 hour intervals. By the

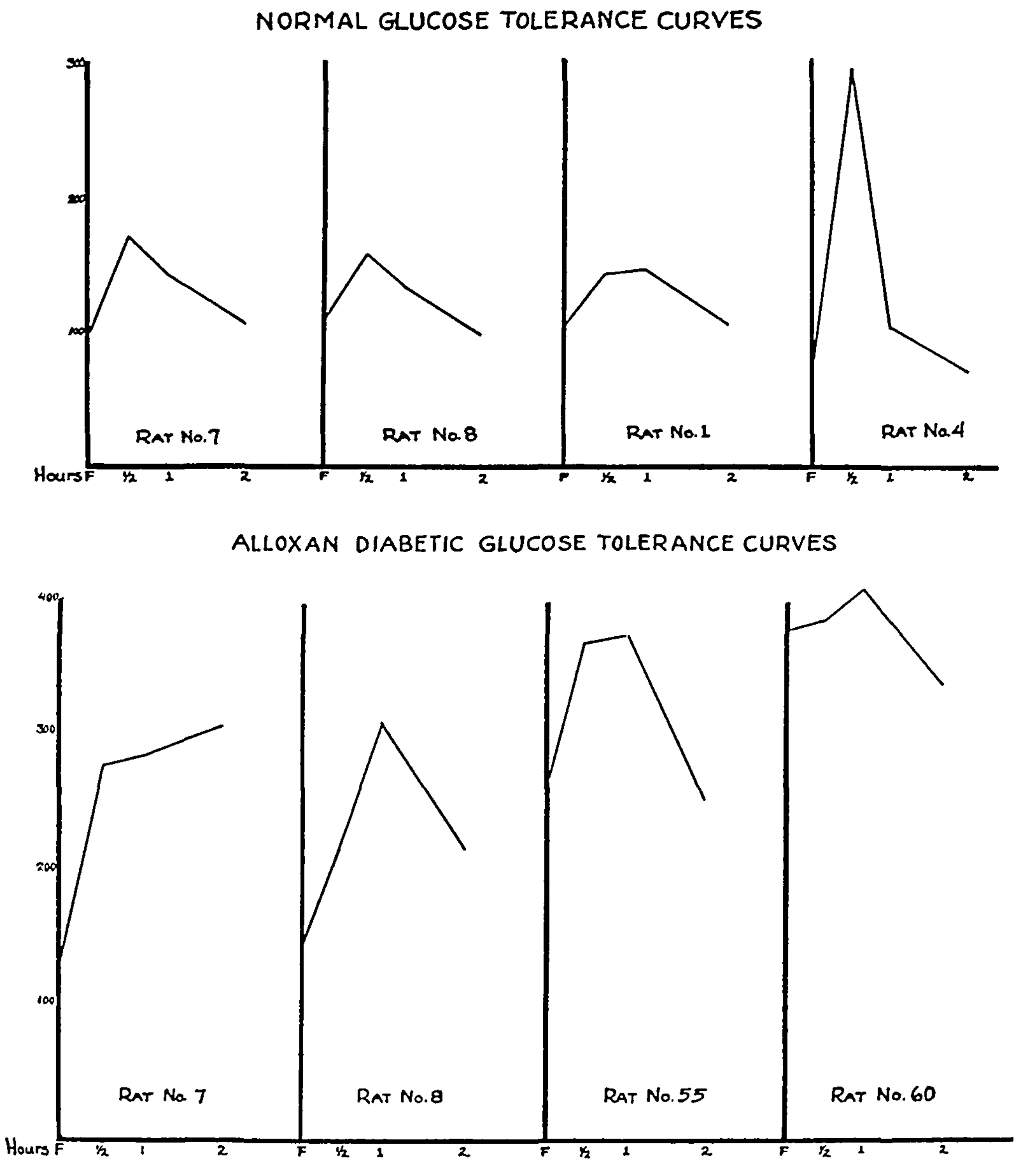



FIG. 1. Comparison of glucose tolerance curves in untreated rats and rats made diabetic by a single intravenous injection of alloxan (30 mg./kg.).

end of 2 hours the blood sugar should return to the fasting blood sugar value, or below, if the rat is normal (Fig. 1). Blood sugars were determined using a modified Folin-Wu procedure (Folin, 1929).

All groups of animals were carried on the experiment until 18 days after the last injection of the 10 dose group. All animals were sacri-

TABLE 1. DATA PRESENTING CORRELATION BETWEEN HISTOLOGY OF THE PANCREATIC ISLETS AND GLUCOSE TOLERANCE IN ALLOXAN-INJECTED AND UNTREATED RATS.

| Group | Number of Animals | Alloxan | Glucose Tolerance | Islets of Langerhan |
|---|---|---|---|---|
| One | 4 | 2 injections 20 mg./kg. | Normal | Normal |
| Two | 10 | 4 injections 20 mg./kg. | Altered | Normal |
| Three | 7 | 10 injections 20 mg./kg. | Altered | Reduction of B* cell number |
| Four | 4 | 1 injection 30 mg./kg. | Altered | Few B* cells present |
| Five | 4 | None | Normal | Normal |

* Beta

TABLE 2. AVERAGES AND RANGES OF BLOOD GLUCOSE LEVELS DURING GLUCOSE TOLERANCE TESTS OF UNTREATED RATS AND RATS RECEIVING MULTIPLE INTRAVENOUS INJECTIONS OF 20 MG./KG. OF ALLOXAN

| No. of Rats | FBS* | ½ hour | 1 hour | 2 hours |
|---|---|---|---|---|
| | | *Untreated Rats* | | |
| 6 | 103 (96–110) | 179 (126–294) | 130 (104–148) | 100 (70–116) |
| | | *2 Injections* | | |
| 4 | 84 (70–104) | 162 (124–190) | 92 (78–108) | 86 (76–94) |
| | | *4 Injections* | | |
| 10 | 98 (80–110) | 195 (156–230) | 167 (140–188) | 145 (132–160) |
| | | *10 Injections* | | |
| 7 | 90 (66–118) | 212 (156–342) | 165 (124–206) | 142 (126–174) |

* Fasting blood sugar.

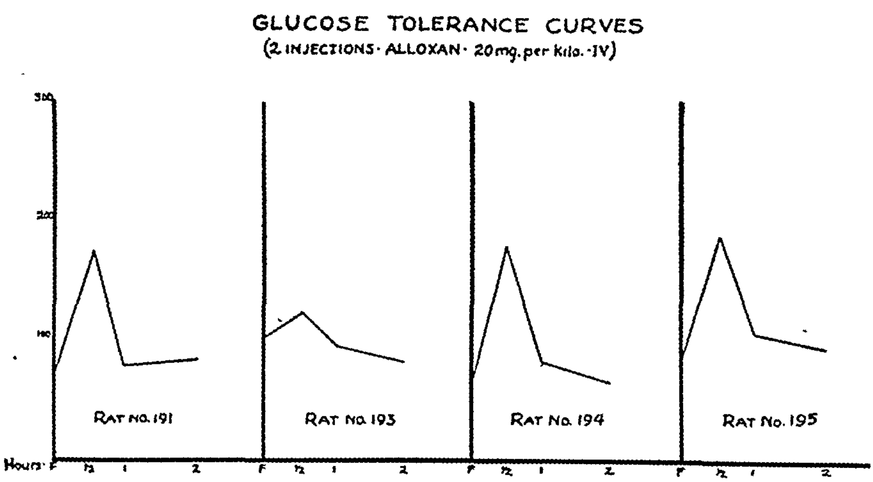



FIG. 2. Glucose tolerance curves of rats receiving two intravenous 20 mg./kg. injections of alloxan. These curves are normal.

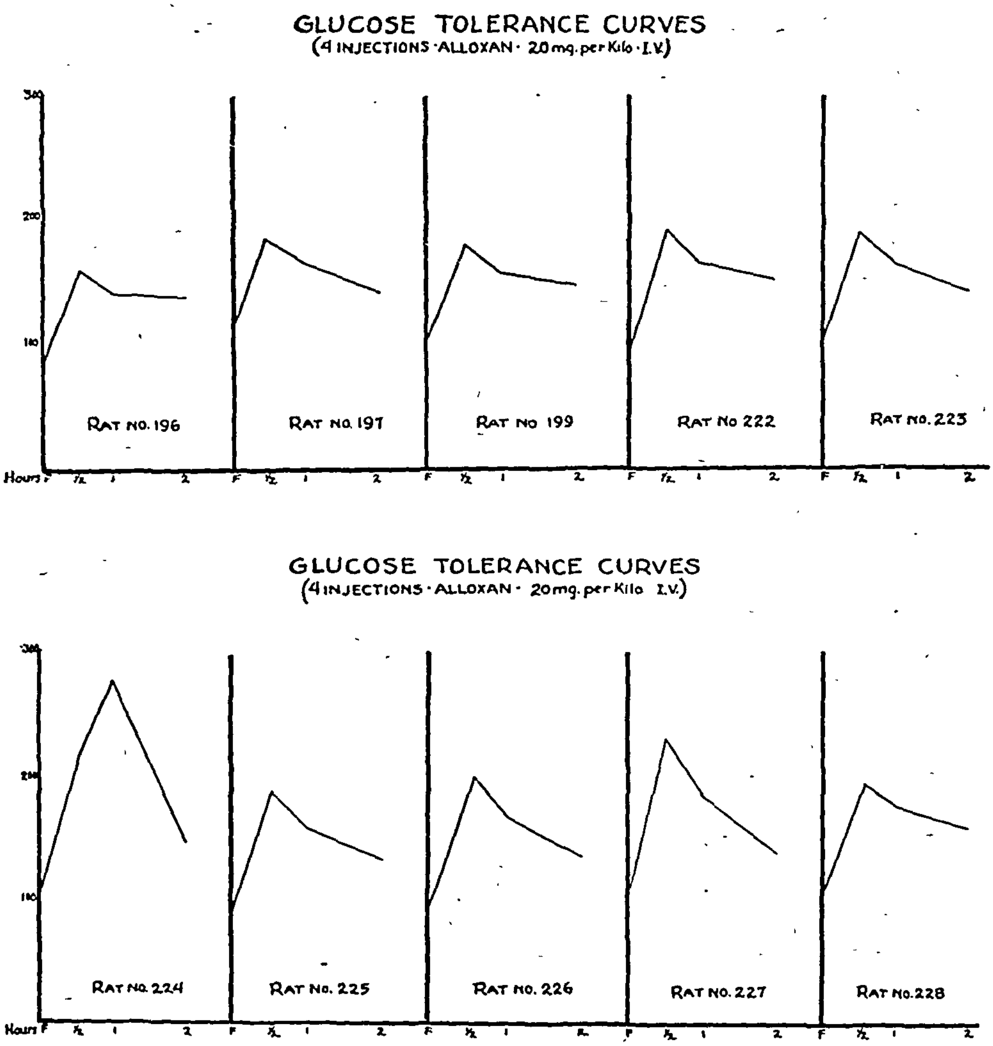



FIG. 3. Altered glucose tolerance curves of rats which received 4 intravenous 20 mg./kg. doses of alloxan.

ficed without starvation at the same time under uniform conditions. Samples of pancreatic tissue were fixed in Zenker's, Bouin's and Lavdovsky's fluid. Tissue was sectioned at 4 microns and stained with Gomori's method (Gomori, 1943) or Heidenhain's iron hematoxylin. Every tenth section was mounted, the alpha and beta cells counted, and the size of the islet noted.

## RESULTS

In animals receiving 2 injections of 20 mg./kg. of alloxan, neither abnormality of glucose tolerance nor what could be considered any significant alteration in the islets of Langerhans was noted (Tables 1 and 2, Figs. 1 and 2). In the group of rats that received 4 injections of 20 mg./kg. of alloxan impairment in glucose tolerance was found

(Fig. 3), the blood level of sugar being higher than normal at the end of 2 hours following the injection of 20 per cent glucose solution. Fasting blood sugar levels were normal in these rats. Microscopic examination of the islets (Figs. 6 and 7) revealed no apparent changes in the beta cells, and no decrease in their number (Table 3) as compared to normal islet tissue. The beta cells appeared to contain their full complement of granules (Fig. 8).

Rats that received 10 injections of 20 mg./kg. of alloxan showed

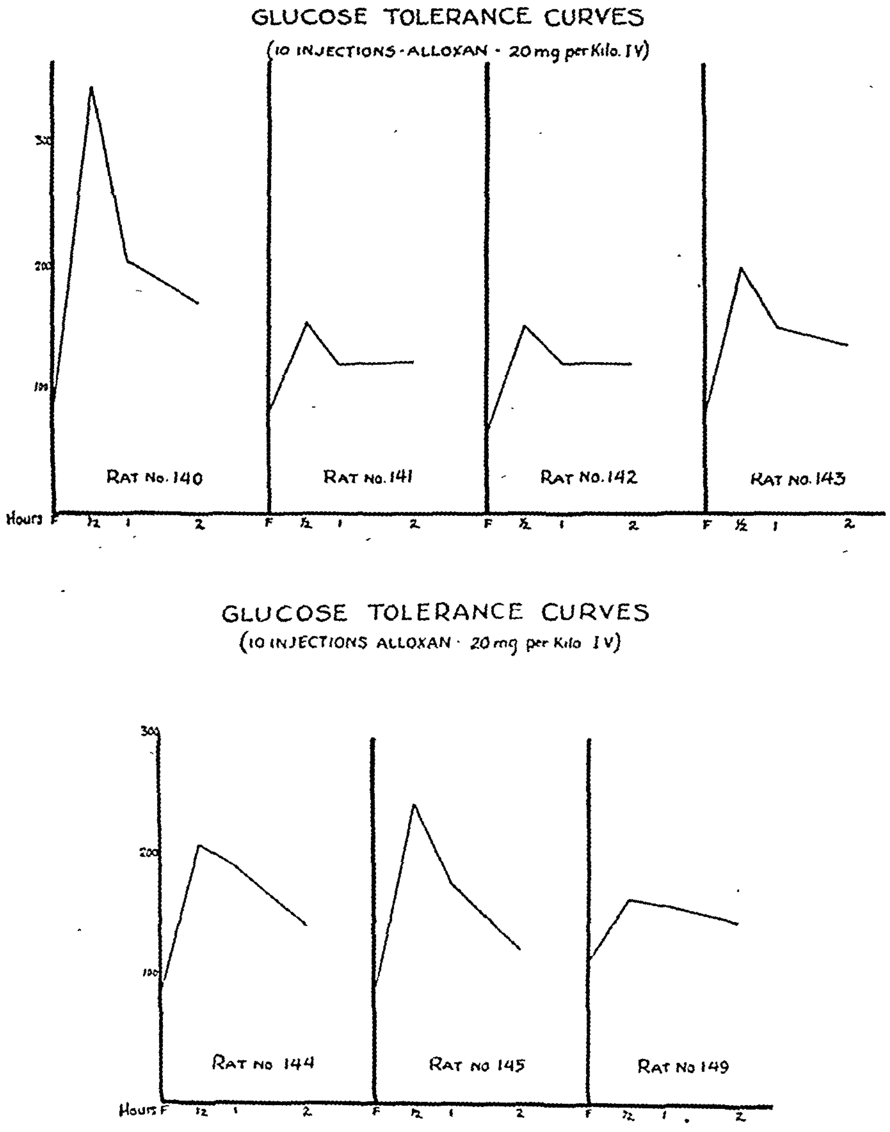



Fig. 4. Altered glucose tolerance curves of rats which received 10 intravenous 20 mg./kg. doses of alloxan.

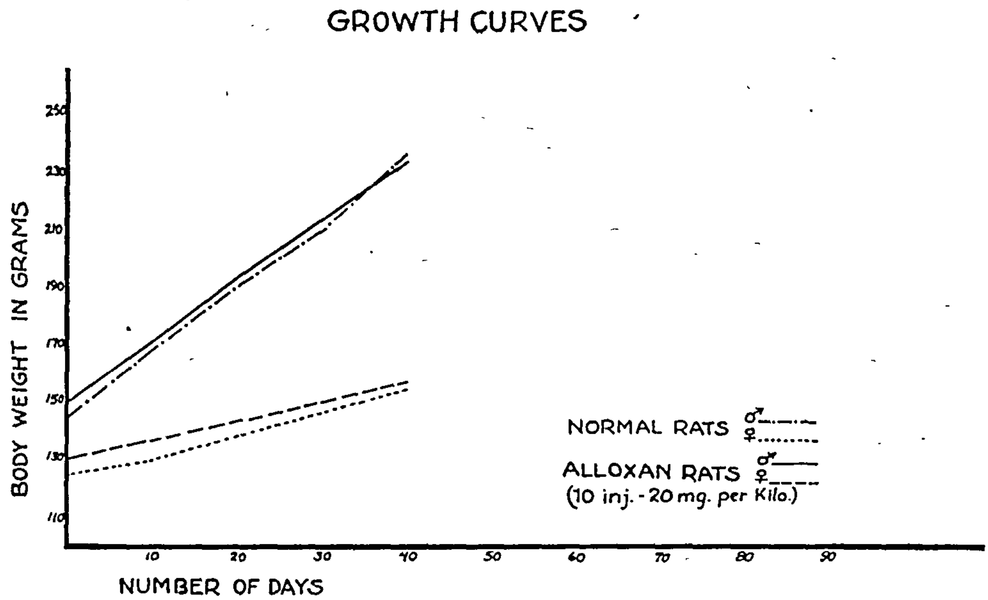



FIG. 5. Comparative growth curves of untreated male and female rats and rats of both sexes which received 10 intravenous 20 mg/kg. doses of alloxan.

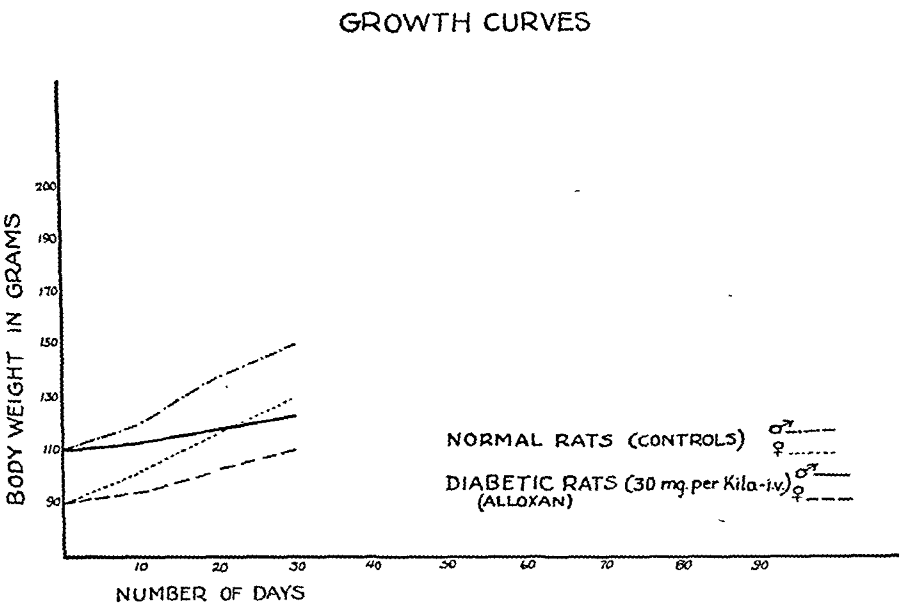
glucose tolerances similar to those found in animals receiving four 20 mg. doses (Fig. 4). In the islets of these animals there was beta cell degeneration (Fig. 9), however, with a reduction in the average number of beta cells for each islet (Table 3). The per cent of beta cells ranged from 0 to 30, as compared to normal islets where there were equal numbers of alpha and beta cells. Hyperglycemia and glucosuria did not occur in the 10 dose animals. Control rats showed normal glucose tolerances (Fig. 1).

The islets of rats receiving a single dose of 30 mg./kg. of alloxan exhibited extensive beta cell degeneration. Some of the islets contained only alpha cells (Fig. 10). These rats showed persistent hyperglycemia and glucosuria, and decidedly altered glucose tolerances (Fig. 1).

TABLE 3. PER CENT DISTRIBUTION OF ALPHA AND BETA CELLS IN THE PANCREATIC ISLET TISSUE OF RATS RECEIVING MULTIPLE INTRAVENOUS INJECTIONS OF 20 MG./KG. DOSES OF ALLOXAN, OR ONE 30 MG./KG. DOSE, OR NO ALLOXAN

| Group | Number of islets | Average per cent distribution of islet cells | |
|---|---|---|---|
| | | Alpha | Beta |
| One<br>2 injections alloxan 20 mg./kg. | 4 | 50 | 50 |
| Two<br>4 injections alloxan 20 mg./kg. | 8 | 50 | 50 |
| Three<br>10 injections alloxan 20 mg./kg. | 8 | 70–100 | 0–30 |
| Four<br>1 injection alloxan 30 mg./kg. | 4 | 70–100 | 0–30 |
| Five<br>Controls | 4 | 50 | 50 |

Diabetic animals (30 mg./kg.) had a stunted growth and a body weight below that of normal rats of the same age (Fig. 5). In the groups receiving 2, 4, or 10 injections of alloxan normal growth was maintained (Fig. 6).



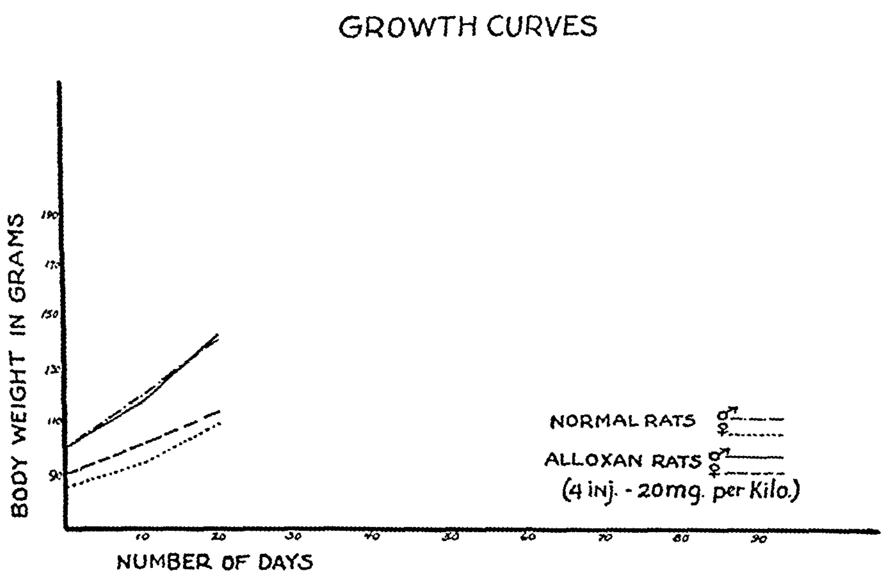




FIG. 6. Comparative growth curves of untreated and alloxan-treated rats. The upper graph compares the growth of normal rats and rats made diabetic by a single intravenous injection of 30 mg./kg. of alloxan. The lower graph compares the growth of normal rats and rats which received 4 intravenous injections of 20 mg./kg. of alloxan.

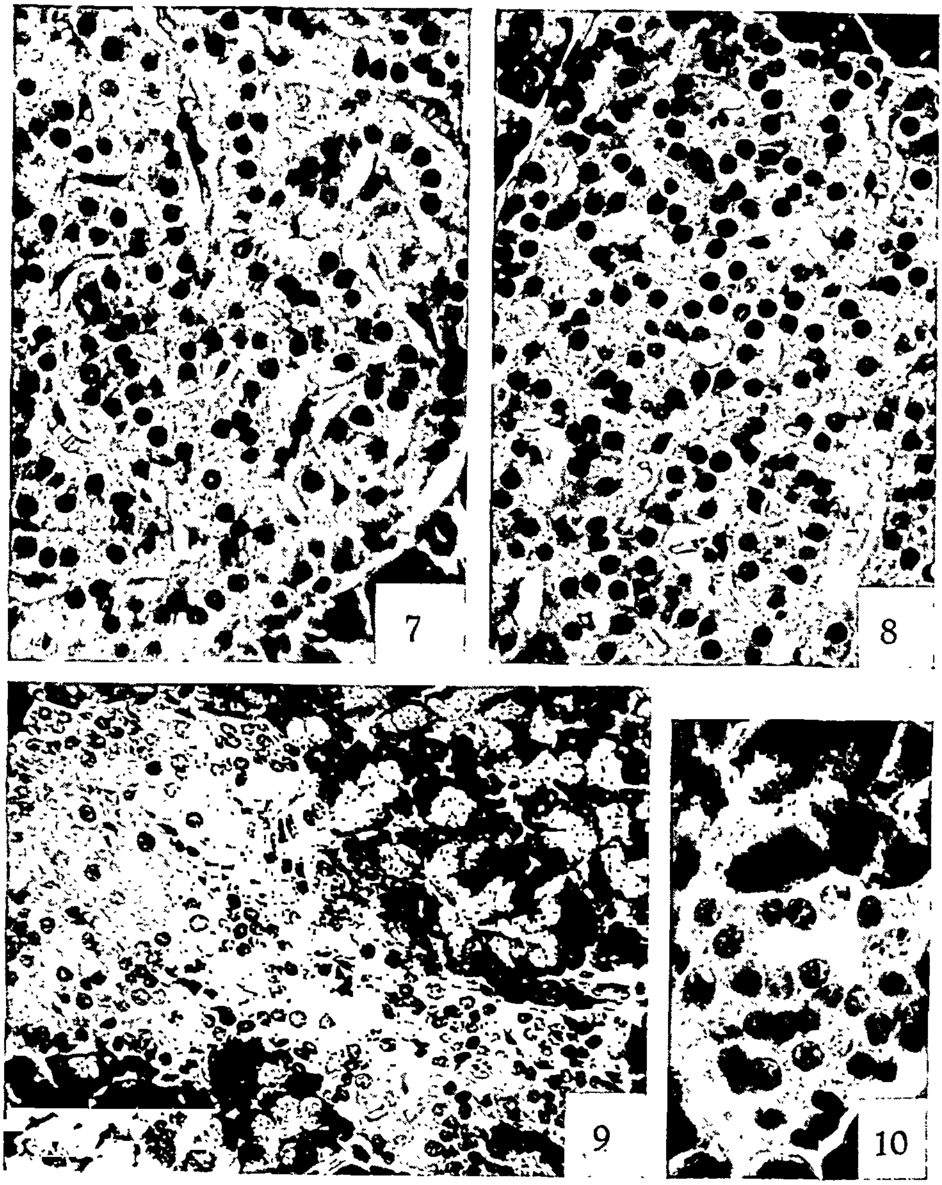



PLATE I

FIG. 7. Islet of Langerhans from an untreated rat. Beta cells compose most of this area, alpha cells being present in only the periphery of the islet in the lower right portion. Lavdovsky-Iron Hematoxylin. Photomicrograph ×300.

FIG. 8. Islet of Langerhans from a rat which received 4 intravenous doses of 20 mg./kg. of alloxan. This animal had an altered glucose tolerance curve. Islet tissue normal. Lavdovsky-Iron Hematoxylin. Photomicrograph ×300.

FIG. 9. Islet of Langerhans from a rat which received 10 intravenous doses of 20 mg./kg. of alloxan. This animal had an altered glucose tolerance curve, but showed neither hyperglycemia nor glycosuria. Growth was normal. Disorganization of islet structure with reduced number of beta cells. Lavdovsky-Delafield's Hematoxylin. Photomicrograph ×150.

FIG. 10. Islet of Langerhans from a rat which received a single intravenous 30 mg./kg. dose of alloxan. This animal was clinically diabetic. Islet small consisting of only alpha cells. Lavdovsky-Iron Hematoxylin. Photomicrograph ×600.

## DISCUSSION

In rats receiving four 20 mg./kg. injections of alloxan, alteration in glucose tolerance without significant histological evidence of beta cell damage was found. Functional alteration of beta cells, that appear intact microscopically, would seem to result from small, repeated doses of alloxan. Animals which exhibit neither hyperglycemia nor glucosuria, but whose beta cells are functionally altered, represent excellent test objects for the influence of other hormones, such as thyroid and adrenal cortical, on the islets. With ten 20 mg./kg. injections of alloxan, although the beta cells were actually reduced in number and the structural alteration was extreme (Fig. 9), glucose tolerance was essentially the same as in the 4 dose animals (Figs. 3 and 4). It is interesting that the rats receiving ten 20 mg./kg. doses were not clinically diabetic, although injection of a single 30 mg./kg. dose resulted in permanent clinical diabetes with degeneration of most of the beta cells (Fig. 10).

The functional disturbance of beta cells in the absence of any decided cytological alteration (with the methods used) that might be considered significant represents a situation which is perhaps analogous to that observed in at least one-fourth of the pancreases of human diabetics (Bell, 1947).

## SUMMARY

Functional alteration of the beta cells of the islets of Langerhans, in the absence of obvious histological damage, resulted from four 20 mg./kg. doses of alloxan given intravenously. The degree of functional inpairment was no greater when, following ten 20 mg./kg. doses, beta cell necrosis was present. Neither treatment induced clinical diabetes, although permanent diabetes ensued following a single intravenous dose of only 30 mg./kg.

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# INSULIN HYPERSENSITIVITY FOLLOWING THE ADMINISTRATION OF DESOXYCORTI-COSTERONE ACETATE[1]

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THE ADMINISTRATION of adrenal cortical hormone inhibits or prevents the release of pituitary adrenocorticotrophic hormone (ACTH) which normally follows subjection of an animal to noxious agents or stressful conditions (Ingle, 1938; Sayers and Sayers, 1947). It has been demonstrated that cortical steroids with an oxygen on $C_{11}$ as well as those of the desoxy type have an inhibitory action upon pituitary adrenocorticotrophic activity. Since these two major types of cortical steroids appear to be qualitatively distinct in their metabolic actions, one group influencing protein and carbohydrate metabolism and the other group electrolyte metabolism, it has been suggested that the cortical steroids act directly on the adenohypophysis rather than indirectly through some product of their metabolic action or through their deficiency (Sayers and Sayers, 1947). A necessary corollary of this "direct action" hypothesis is the following: Administration of desoxycorticosterone acetate (DCA), by inhibiting the adrenocorticotrophic activity of the adenohypophysis and in turn the secretory activity of the adrenal cortex, should lead to a disturbance in carbohydrate metabolism characteristic of a deficiency of those steroids having O on C-11. The hypothesis is supported by the experiments presented in this paper which demonstrate that DCA-treated rats are more sensitive than untreated animals to the hypoglycemic action of insulin.

## MATERIALS AND METHODS

Male rats from the Sprague-Dawley farm, weighing 220 to 450 grams, were employed. In order to demedullate the rats, the adrenals were enucleated through paravertebral incisions. Before DCA was implanted, a period of two weeks was allowed for regeneration of the cortex from the capsule in series I and II, and a period of one week in series III. Each series was divided equally into two groups; one group served as a control and into each member of the other group six 15-mg. pellets of DCA were implanted subcutaneously. The pellets were distributed over the region of the flanks. The amount of DCA

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[1] Supported by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

[2] Fellow of the American Bureau for Medical Aid to China.

absorbed was calculated from the difference between the original weight of the pellets and their weight at the time of autopsy. The average amount absorbed per rat per day was 1.7 mg.

The insulin sensitivity tests were conducted from the second to fourth weeks after the DCA implantation. The animals were fasted 12 hours and then regular insulin was administered intraperitoneally in a dose of 0.5 unit per Kg. Blood samples for sugar analysis were taken from the tail vein immediately preceding the administration of insulin and at hourly intervals thereafter for four hours. Blood sugars were analyzed by Nelson's (1944) modification of the Somogyi method.

The aqueous[3] and lipid[4] extracts of adrenal cortex were administered intramuscularly. The ethanol preservative was removed from the aqueous extract just preceding its administration. At the time of the insulin test each rat was given cortical extract in two equal doses; the first injection was made one-half to one hour before and the second injection immediately following the administration of insulin. Each single dose of the lipid extract was one to 1.25 ml. and each single dose of aqueous extract was the equivalent of 2.5 ml. of the original aqueous-alcohol solution.

The experiments were planned so that an equal number of controls and treated animals were tested for insulin sensitivity on any one particular day. Usually three controls and three DCA-treated rats were tested on the same day. Each experiment designed to test the therapeutic effectiveness of cortical extract in DCA-treated animals included two controls, two DCA-treated rats and two DCA cortical extract-treated rats.

Additional experiments were performed on completely adrenalectomized rats in order to obtain an estimate of the relative sensitivity of the DCA-treated demedullated rats to insulin.

## RESULTS

*Insulin sensitivity of DCA-treated demedullated rats.* The results of series I, a total of 19 rats, are presented in Figure 1. The data for series II and III, a total of 22 rats, have been combined in Figure 2. The dose of insulin used, 0.5 unit per Kg., is smaller than that required to produce the same reduction in blood glucose in rats with intact adrenal medullas. Demedullated rats were employed in order to avoid the complication of epinephrine discharge which occurs after insulin administration.

There was no significant difference between the fasting blood glucose levels of the untreated demedullated rats and the DCA-treated demedullated rats. This was true of all three series of animals.

It is apparent from Figure 1 (series I) and Figure 2 (series II and III) that administration of DCA to demedullated rats markedly increases their sensitivity to insulin. In series I, five of the 10 DCA-treated animals died in hypoglycemic coma; the remaining five had insulin tolerance curves which clearly indicated increased sensitivity to insulin. None of the control animals died. The tolerance curve of

[3] Adrenal Cortex Extract (Upjohn).

[4] Lipo-Adrenal Cortex (Upjohn).

only one of the nine control animals was in the zone of the curves of the DCA-treated animals. The incidence and severity of coma and convulsions in the surviving DCA-treated animals were greater than in the untreated controls. One of the rats of the DCA group died at a time when the blood sugar level was above what might be considered critical; the explanation of this is not apparent.

In series II and III (data combined in Figure 2), the results confirm those obtained in series I. Three of the 8 DCA-treated rats died

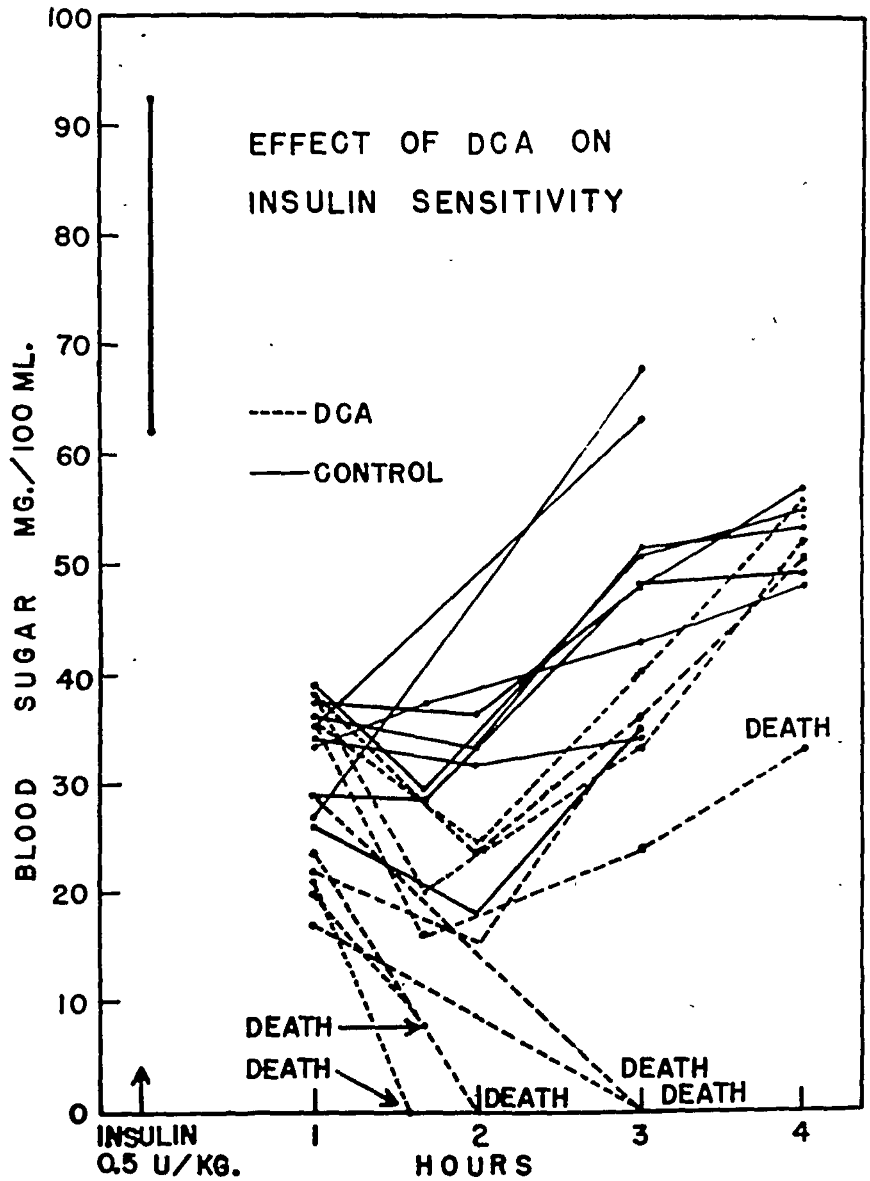


FIG. 1. Series I. The effect of DCA administration in demedullated rats upon insulin sensitivity. Regular insulin was injected intraperitoneally in a dose of 0.5 unit per Kg. at zero time. The vertical line at zero time represents the range in fasting blood sugar of all rats in series I.

in hypoglycemic coma following insulin administration whereas no deaths occurred in the control group. The tolerance curves of the two groups overlapped but slightly. Again the incidence and severity of convulsions and coma were greater in the surviving DCA-treated animals than in the controls. From Figure 2 it can be seen that cortical extract increased the resistance of the DCA group to insulin to

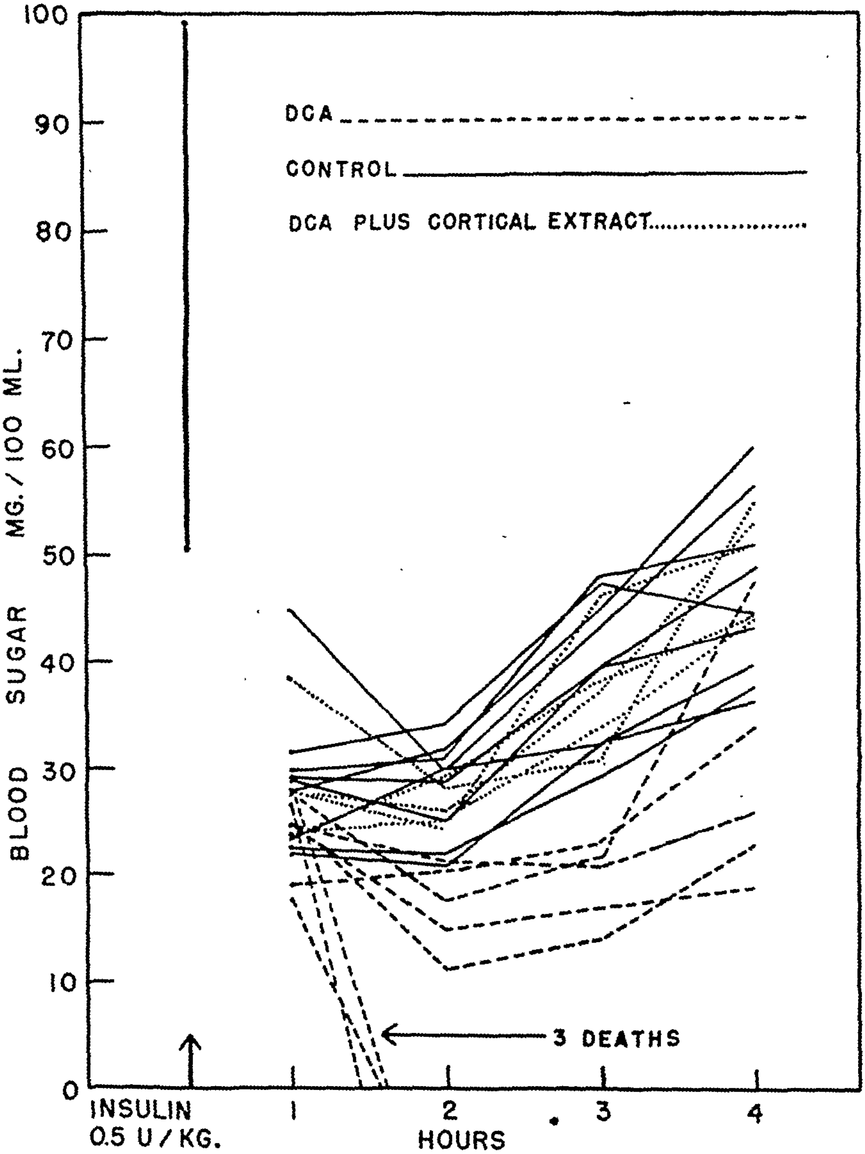


FIG. 2. Series II and III. The effect of DCA administration in adrenal demedullated rats upon insulin sensitivity; the antagonistic action of cortical extract. Regular insulin was injected intraperitoneally in a dose of 0.5 unit per Kg. at zero time. The vertical line at zero time represents the range in fasting blood sugar of all rats in series II and III.

such a degree that their tolerance curves fell into the normal zone. Furthermore, the clinical symptoms of the animals given DCA and cortical extract were indistinguishable from those of the demedullated controls.

*Effect of adrenalectomy on insulin sensitivity.* An estimate was made of the relative sensitivity of the DCA group to insulin in comparison

TABLE 1. RELATIVE INSULIN SENSITIVITY OF ADRENALECTOMIZED, DEMEDULLATED AND DCA TREATED-DEMEDULLATED RATS

| Dose of insulin units per Kg. | Group | Blood sugar mg. per 100 ml. | | | | |
|---|---|---|---|---|---|---|
| | | Fasting level | Hours after insulin administration | | | |
| | | | 1 | 2 | 3 | 4 |
| 0.25 | demedullated | 67 | 31 | 35 | 44 | 63 |
| | | 59 | 32 | 32 | 40 | 51 |
| | demedullated plus DCA | 54 | 38 | 42 | 43 | 50 |
| | | 55 | 23 | 20 | 30 | 42 |
| | adrenex | 48 | 24 | 18 | 16 | 22 |
| | | 62 | 24 | 18 | 25 | 34 |
| 0.50 | demedullated | 83 | 30 | 31 | 48 | 51 |
| | | 75 | 31 | 34 | 47 | 45 |
| | demedullated plus DCA | 70 | 28 | Died | | |
| | | 65 | 28 | Died | | |
| | adrenex | 64 | 9.5 | Died | | |
| | | 66 | 22 | Died | | |
| 1.00 | demedullated | 68 | 11 | 5 | 6 | Died |
| | | 70 | 19 | 19 | 50 | 51 |
| | | 74 | 24 | 19 | 21 | 34 |
| | | 60 | 23 | 21 | 26 | 34 |
| | | 62 | 15 | 25 | 18 | 20 |
| | demedullated plus DCA | 71 | 15 | Died | | |
| | | 66 | 12 | Died | | |
| | | 62 | 15 | Died | | |
| | | 62 | 19 | Died | | |
| | | 55 | 16 | — | 17 | 21 |
| | adrenex | 71 | 4 | Died | | |
| | | 69 | 2 | Died | | |
| | | 65 | 8 | Died | | |
| | | 63 | 17 | Died | | |

with that of completely adrenalectomized animals. Table 1 presents the results of this study. All members of the three groups,—the demedullated control, the demedullated DCA-treated, and the adrenalectomized animals,—survived following the administration of 0.25 unit of insulin per Kg. However, the tolerance curves suggest that the adrenalectomized rats were slightly more sensitive than the DCA-treated animals. Only the controls survived the administration of 0.5 unit of insulin per Kg. Following the administration of 1.0 unit of insulin per Kg. only one member of the control group died, whereas

all the DCA-treated animals succumbed except one; all four adrenalectomized rats died following such treatment. Again the tolerance curves indicate that the adrenalectomized animals are more sensitive to insulin than are the DCA-treated rats.

## DISCUSSION

Demedullated rats were employed in these studies in order to eliminate the possible masking of insulin sensitivity by discharge of epinephrine from the adrenal medulla. The cortex rapidly regenerates from the capsule following enucleation of the adrenal and when regeneration is complete the animal appears to be normal except for its response to those stressful situations in which the elaboration of epinephrine represents an essential phase of the attempt to maintain homeostasis (see Ingle [1944] for a discussion of the problem of the resistance of the adrenal demedullated animal).

The results of this study clearly indicate that the administration of DCA produces an increase in the sensitivity of rats to insulin. The degree of sensitivity approaches but is not quite as severe as that of the adrenalectomized animal. It is reasonable to suppose that the increased sensitivity to insulin which follows administration of DCA is a result of a relative deficiency of cortical steroids having an O on C-11. The evidence is admittedly indirect but the explanation is in accord with our present knowledge of the influence of hormones upon carbohydrate metabolism. A number of published reports have appeared which may also be interpreted to indicate that DCA produces an impairment of carbohydrate metabolism characteristic of a deficiency of those steroids with an oxygen on C-11. For example, administration of DCA is said to increase the tolerance of diabetic patients to glucose (Köhler and Fleckenstein, 1941, 1942; Winnett *et al.*, 1940). McGavack *et al.* (1941) have demonstrated that DCA influences the response of normal human subjects to oral glucose; the administration of DCA caused "an early higher rise and a marked depression approaching hypoglycemic levels in the fourth and fifth hour." Furthermore, chronic treatment with DCA impairs the ability of the rat to mobilize glucose when subjected to a variety of nonspecific types of stress (Selye and Dosne, 1942).

The effect of DCA upon electrolyte metabolism has been definitely established. Unfortunately, it is not possible at the present time to reach a final conclusion regarding the influence of this steroid on carbohydrate metabolism. Does its action differ in degree only from those steroids with an O on C-11, or is DCA qualitatively distinct in its metabolic activity? The answer to this question has an important bearing on the interpretation of the results which have been presented in this paper. Under the conditions of these experiments DCA acts as an "antidiabetogenic" substance. Furthermore, even in large doses DCA has no activity as measured by the glycogen deposition test

(Olson *et al.*, 1944; Pabst *et al.*, 1947). However, two groups of investigators have reported that DCA has a "diabetogenic" action. Ingle and Thorn (1941) found that doses of 1, 2 or 5 mg. per day of 17-hydroxy-11-dehydrocorticosterone were followed by enhanced glycosuria in partially depancreatized rats, whereas similar doses of DCA had no effect. However, 10 mg. of DCA did produce a definite exacerbation of the glycosuria. Wells and Kendall (1940) demonstrated that DCA, although not quite as effective as Compound E, very definitely increased the glycosuria of the phlorhizinized-adrenalectomized rat. These experiments support the view that large doses of DCA have a diabetogenic effect, *i.e.*, a metabolic action similar to that of the cortical steroids with an O on C-11. However, it is important to note that this so-called "diabetogenic" action has been demonstrated using large doses of DCA in experiments employing glycosuria as an index of "diabetogenic" action. It is possible that large doses of DCA produce this "diabetogenic" effect through a renal mechanism, that is, by inhibiting the reabsorption of glucose by the renal tubule. This possibility takes on added significance in the light of the experiments of Wells and Kendall (1940) who noted that Compound E markedly increased the glycosuria of phlorhizinized-adrenalectomized rats and at the same time prevented the collapse, convulsions and death which frequently occurred in the untreated controls. On the other hand, DCA produced a definite although less marked glycosuria, but was entirely ineffective in preventing coma, convulsions and death. This may be interpreted to mean that DCA did not benefit the adrenalectomized animals by elevating the blood sugar but rather acted synergistically with phlorhizin to inhibit reabsorption of glucose at low blood sugar levels. This possibility is currently being subjected to experimental test by renal clearance studies.

It is obvious that further experimental work will be necessary in order to establish definitely whether DCA has any action at all on carbohydrate metabolism similar to that of the steroids with an O on C-11. The results of the least complicated test, namely, the glycogen deposition test, would seem to indicate that DCA is qualitatively distinct in its metabolic actions from those steroids with an O on C-11.

It has been demonstrated that the administration of cortical hormones prevents or inhibits the discharge of ACTH which otherwise follows subjection of an animal to a variety of nonspecific types of stress (Sayers and Sayers, 1947). It would appear reasonable to suppose that the great variety of non-specific stresses increases pituitary adrenocorticotrophic activity by a common mechanism, namely, by increasing the requirement of the peripheral tissue cells for cortical hormones. Since the inhibitory effect on the pituitary is exerted by both the desoxy type cortical steroids and those having an O on C-11,

steroids which differ markedly or even qualitatively in their metabolic actions, it has been suggested that the steroids act directly on the adenohypophysis, rather than indirectly through some product of their metabolic activity or through their deficiency. A corollary follows from this "direct action" hypothesis. Administration of DCA, by suppressing pituitary adrenocorticotrophic activity and in turn the secretory activity of the adrenal cortex, should result in a cortical hormone imbalance characterized by an excess of DCA and a deficiency of those normally secreted cortical steroids which influence carbohydrate metabolism. The experiments presented in this paper support such a view.

The concept of "direct action" of cortical steroids on the adenohypophysis appears to us to be the most reasonable explanation of the experimental facts at present available. However, other interpretations are possible. For example, DCA might produce the effect described by a direct inhibitory action on the secretory activity of the adrenal cortex. Although evidence in favor of such an action has been presented by Greep and Deane (1947), the experimental results of Ingle *et al.* (1938) and Sayers and Sayers (1947) are against such a possibility.

The structural similarity of desoxycorticosterone and the steroids with an oxygen on C-11 presents the possibility of competitive inhibition. It is conceivable that these two types of steroids could interfere with each other in their actions upon a specific target cell. However, the fact that the administration of DCA causes atrophy of the adrenal cortex (Villela, 1943) is strong evidence in favor of an action, direct or indirect, through the adrenal cortex itself.

The authors in collaboration with Dr. Dixon Woodbury have recently demonstrated that the elevation of both the electroshock seizure threshold and the plasma sodium of intact rats treated with DCA can be reduced to normal levels by ACTH administered concomitantly with the DCA. These results may be interpreted to mean that at least part of the action of DCA in the intact animal is a result of the suppression of pituitary adrenocorticotrophic activity with a consequent reduction in the secretory activity of the adrenal cortex. They lend support to the concept of "direct action" presented above.

Selye and Pentz (1943) have demonstrated that DCA administered in large doses to rats on a high sodium chloride intake causes hypertension, nephrosclerosis, cardiac lesions and periarteritis nodosa, and believe these experiments support their concept that certain chronic degenerative diseases in man are a result of hypercorticism. However, according to the present study, DCA treatment leads to the development of a state of steroid hormone imbalance characterized by an excess of a compound influencing electrolyte metabolism and a deficiency of steroids with an O on C-11. The role of the deficiency state as well as that of the excess DCA must be con-

sidered in arriving at conclusions regarding the etiological role of DCA in the production of pathological conditions of the cardiovascular system.

SUMMARY

The administration of DCA to adrenal demedullated rats resulted in insulin hypersensitivity. The DCA-treated animals were more sensitive than untreated controls but less sensitive than adrenalectomized rats. The results have been interpreted to mean that DCA produces a state of relative deficiency of those cortical steroids with an O on C-11. A number of possible mechanisms whereby DCA can affect insulin sensitivity has been presented. A direct inhibitory action of DCA on pituitary adrenocorticotrophic activity appears to be the most reasonable explanation of the facts at present available.

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# A NEW PROCEDURE FOR THE DETERMINATION OF THE ANTIDIURETIC PRINCIPLE IN THE URINE[1]

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IT IS GENERALLY accepted that the hormone of the neurohypophysis secretes a hormone which plays an important role in the water economy of the organism. This antidiuretic hormone is presumably excreted in part in the urine, the antidiuretic activity of which has been found to parallel the need of the organism for the hormone (Gilman and Goodman, 1937). Thus during dehydration resulting from forced abstinence from water or in various clinical conditions (cirrhosis of the liver with ascites (Ralli, *et al.*, 1945), the nephrotic stage of glomerulonephritis (Robinson and Farr, 1940), eclampsia (Ham and Landis, 1942,) etc.), the antidiuretic potency of the urine, is enhanced considerably over normal. In dehydration, structural changes are also noted in the neurohypophysis, which are suggestive of increased activity of this tissue (Gersh, 1939).

The available methods for the determination of the antidiuretic principle in the urine are laborious. Although the hydrated rat is very sensitive to small doses of the hormone, losses are apt to occur in the available procedures used in concentrating the urine in the commonly used method of Burn (1931) and its modifications. These procedures consist in concentrating 24-hour collections of urine by evaporation and dialysis of the residue. However, the available evidence (Ham and Landis, 1942; Ralli, *et al.*, 1945; Donaldson, 1947) indicates that the membranes used for the latter procedures are not entirely impermeable to the hormone which may, therefore, be partially lost.

The present paper describes a procedure for concentrating the hormone in urine which is simple and does not require dialysis. The hormone derived from larger volumes of urine may be concentrated in the final extract with the exclusion of toxic urinary constituents which is not possible by previous methods. It is thus possible to detect even small amounts of the antidiuretic hormone by concentrating correspondingly larger volumes of urine.

Posterior pituitary solutions manifest a chloruretic as well as an

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antidiuretic action. The antidiuretic action of urinary concentrates, on the other hand, is not accompanied by a corresponding chloruretic effect which might be taken as evidence against the identity of the hormone with the principle occurring in the urine. The probable cause of this anomaly has also been elucidated.

## METHODS

For comparative purposes, the procedures used by previous workers for extracting the hormone were utilized. Twenty-four-hour collections of urine were acidified with 3 per cent acetic acid to about pH 6, placed in Visking membranes and concentrated at room temperature by suspension in the air under an electric fan. The concentrated urine was then dialyzed for 6 hours and evaporated further to a volume of about 80 cc. This residue, after filtration, was brought to a volume of exactly 100 cc. with distilled water and injected in doses of 1 cc. per 100 gm. of body weight into the test rats. The bioassay procedure of Burn (1931) as modified by Ralli, *et al.* (1945) was used. For each determination, 6 groups of 3 rats were used, 2 groups serving as controls.

In the modified procedure which has been devised, the sample of freshly collected urine is filtered and brought to pH 4.5 to 5.0 by the addition of acetic acid. One gm. of absorbent powdered charcoal (Darco activated carbon, Grade G-60) is added for each 100 cc. of urine and the mixture agitated at intervals for several hours. It is placed in the ice-chest overnight and the supernatant urine discarded. The charcoal is collected on a Buchner funnel, washed with small amounts of distilled water to remove adherent urine, drained and transferred to a centrifuge tube. The charcoal on which the antidiuretic hormone is quantitatively adsorbed, is suspended in glacial acetic acid (5 cc. per gm. of charcoal)and agitated at intervals for several hours. The acid is separated from the charcoal by centrifugation or filtration and the hormone and other adsorbed materials precipitated by the addition of 10 volumes of a mixture of equal parts of absolute ethyl alcohol and petroleum ether. This precipitate is removed by centrifugation, the supernatant fluid being discarded. After drying in a vacuum desiccator at room temperature for at least one hour to remove the organic solvents, the precipitate is dissolved in the requisite amount of distilled water, filtered and bioassayed as indicated above.

The chloruretic action of the final extract was determined by analysis of the chloride content of the urine (50 per cent output) excreted by the rats used for assay. Chloride was determined by Stiff's (1948) modification of Sendroy's method or by the procedure of Van Slyke (1923).

## RESULTS

The procedure for determining the antidiuretic activity of the urine as outlined above is based on the fact that the antidiuretic principle is adsorbed quantitatively on charcoal, eluted from the latter by glacial acetic acid and precipitated from the last-named by petroleum ether and absolute alcohol. The solubility of the antidiuretic factor in glacial acetic acid and its precipitation by petroleum ether was originally demonstrated by Kamm *et al.* (1928). By treating solu-

tions of commercial posterior pituitary extract by the above-outlined procedures, good recovery of the active principle is obtained as shown in Table 1. The data of this table were obtained by diluting commercial posterior pituitary extract with 0.9 per cent saline to give concentrations varying from 20 to 100 milliunits per cc. as shown in column 1. Aliquots of these solutions were bioassayed for their antidiuretic activity as described above in doses of 1 cc. per 100 gm. of body weight of the test rats. Similar assays were also performed on the final solutions obtained after subjecting an aliquot of the original diluted extracts to the adsorption-elution procedure outlined above. The second

TABLE 1. THE RECOVERY OF ANTIDIURETIC ACTIVITY FROM SOLUTIONS OF COMMERCIAL POSTERIOR PITUITARY LIQUID BY ADSORPTION ON CHARCOAL, ELUTION WITH GLACIAL ACETIC ACID AND PRECIPITATION WITH PETROLEUM ETHER

| Concentration of posterior pituitary solution | Delay Times | |
|---|---|---|
| | Original extract | After adsorption, elution, etc. |
| milliunits per cc. | mins. | mins. |
| 20 | 20 | 20 |
| 30 | 40 | 30 |
| 40 | 50 | 50 |
| 50 | 55 | 50 |
| 60 | 65 | 65 |
| 70 | 90 | 95 |
| 80 | 105 | 100 |
| 100 | 130 | 125 |

and third columns of the table give the delay times in the excretion of the water of the test rats. This delay time is the difference in time required to excrete half the water administered by the rats receiving extract as compared to the control animals receiving saline or water injections.

The method just described reduces the possibility of loss of the active principle by dialysis. Previous authors have obtained divergent results in their study of the diffusibility of the active principle (Ham and Landis, 1942; Ralli, *et al.*, 1945; Donaldson, 1947). Our own experiments using ultra-filtration and dialysis through cellophane membranes are reproduced in Table 2. The data of this table were obtained by dialyzing a solution of commercial posterior pituitary liquid containing 20 milliunits per cc. for 6 hours against running tap-water or ultrafiltering the solution under a pressure of 100 mms. of Hg through a Visking membrane. As noted in Table 2, there was only a slight loss of antidiuretic activity on prolonged dialysis. However, as seen in the results obtained on the ultrafiltrate, the membrane was definitely permeable to this principle as well as to the chloruretic factor. It would appear, therefore, that the active principle is of such a molecular size as to be on the border line as regards its capacity to pass pass through the available membranes. This would account for the divergent results of previous workers who have studied the dialyza-

bility of the active principles of posterior pituitary extracts and urine (Smith and McClosky, 1924; Gilman and Goodman, 1937; Donaldson, 1947). In any case, the avoidance of the necessity of dialysis avoids the possibility of loss of the hormone. Another advantage of the present method is that it permits one to concentrate much larger amounts of urine without undue toxicity of the final extract which is not the case when evaporation and dialysis are used, as in previous methods. As shown in the subsequent paper, the method permits the demonstration of antidiuretic activity in normal urine as well as in conditions in which its concentration is greatly increased.

TABLE 2. THE EFFECT OF DIALYSIS ON THE ANTIDIURETIC AND CHLORURETIC ACTIVITY OF AN AQUEOUS SOLUTION OF COMMERCIAL POSTERIOR PITUITARY LIQUID CONTAINING 20 MILLIUNITS PER CC.

| | Chloride excretion | Time for 50 per cent urinary excretion |
|---|---|---|
| | m.eq. per 100 g. | mins. |
| Controls (saline) | 1.4<br>1.5 | 90<br>95 |
| Original solution of posterior pituitary liquid | 5.4<br>5.9 | 165<br>150 |
| Same after dialysis for 6 hours | 2.5<br>1.5 | 155<br>120 |
| Ultrafiltrate of same | 7.3<br>6.3 | 115<br>130 |

*The Chloruretic Action of Posterior Pituitary Extract*

As pointed out by previous workers, the principle responsibile for the antidiuretic action of urine differs in notable respects from that of posterior pituitary solution which in turn differs in action from that of the press-juice of fresh posterior pituitary glands (Ham and Landis, 1942). The most notable difference between the extract or the press-juice and the principle in the urine is the absence in the latter of the characteristic chloruretic activity manifested by the glandular preparations. This has led many observers to question the view that the urinary principle is derived from the pituitary. It is now recognized, however, that the hormone of the posterior pituitary is a single molecule which can be readily converted to moieties of the molecule which are still capable of manifesting part of the action of the original hormone (literature reviewed by Grollman, 1947). It appeared to us likely, therefore, that one could explain the lack of chloruretic action of the urinary principle by assuming that the original hormone was partially altered in the organism, perhaps through oxidation. We, therefore, oxidized commercial posterior pituitary solution containing 10 units per cc. and saline extracts of fresh glands by adding about 1 cc. of 95 per cent hydrogen peroxide to 10 cc. of extract. After allow-

ing the mixture to stand for several hours, the solution was heated to boiling and zinc dust added to destroy the excess peroxide. The resulting solution after dilution with normal saline to give a solution containing 20 milliunits per cc. was tested for its chloruretic and antidiuretic action. As shown in Table 3, the chloruretic effect is lost following oxidation without appreciable loss of antidiuretic action. These findings support the view that a similar effect occurs in the body and that the antidiuretic principle of urine, despite its lack of chloruretic activity, may be an altered product of the posterior pituitary hormone.

TABLE 3. THE EFFECT OF OXIDATION ON THE CHLORURETIC AND ANTIDIURETIC ACTIVITIES OF POSTERIOR PITUITARY SOLUTION

| | Chloride excretion | Time for 50 per cent urinary excretion |
|---|---|---|
| | m.eq. per 100 g. | mins. |
| Controls (saline) | 0.6 | 90 |
| | 1.8 | 100 |
| | 1.6 | 80 |
| Untreated posterior pituitary solution | 10.3 | 160 |
| | 8.6 | 180 |
| | 12.0 | 165 |
| Oxidized posterior pituitary solution | 1.2 | 180 |
| | 0.9 | 160 |
| | 1.7 | 165 |

The results of Table 3 were obtained on commercial posterior pituitary liquid. Comparable results were obtained on saline extracts of fresh glands and on extracts of desiccated posterior pituitary powder.

## DISCUSSION

The procedure outlined is not only simpler and more convenient but also affords a more accurate method for the concentration of the antidiuretic principle from urine than those previously available. It has been applied to a study of hypertension as shown in the following paper with results not obtainable by the older procedures of concentration by evaporation and dialysis.

The results of the present paper on the destruction of the chloruretic action of the hormone by oxidation removes one of the chief objections hitherto raised against the view that the antidiuretic principle in urine is derived from the posterior pituitary hormone. The ease with which the hormone as it occurs in fresh press-juice is altered in its properties by chemical manipulation (Van Dyke, *et al.*, 1942) and split into pressor and oxytocic fractions and the rapidity with which injected extracts disappear from the blood stream are evidence of the lability of the hormone. One would anticipate, therefore, that the excretory product of the hormone might appear in an altered form differing in many respects from the native hormone.

The results of the present study support the view that the chloruretic action of posterior pituitary extract is due to a specific action of the hormone (Silvette, 1940; Little, *et al.*, 1947), as opposed to the view that this action is merely incidental to its antidiuretic action.

## SUMMARY

A new procedure is described for concentrating the antidiuretic principle of the urine based on adsorption on charcoal, elution by glacial acetic acid and precipitation by petroleum ether and absolute alcohol. The method offers several advantages over previously available procedures.

Oxidation of posterior pituitary extract was shown to result in a loss of its chloruretic with retention of its antidiuretic activity. It is suggested that a similar reaction occurs in the organism and that this may account for the lack of chloruretic action by the antidiuretic principle of the urine.

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# THE ANTIDIURETIC HORMONE IN THE URINE IN EXPERIMENTAL AND CLINICAL HYPERTENSION[1]

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Hypertension, experimentally induced in laboratory animals as well as the clinical form of this disorder, is accompanied by certain changes in salt and water metabolism (Grollman, 1947). These observations suggested the desirability of a study of the excretion of the antidiuretic principle in the urine in various forms of hypertension in order to determine to what extent the altered salt and water metabolism in hypertension is accompanied by changes in the antidiuretic activity of the urine.

## METHODS

The procedures outlined in the preceding paper were utilized for the determination of the antidiuretic content of the urine. Twenty-four hour specimens of urine were collected from a group of patients suffering from hypertensive cardiovascular disease who had been followed in the hypertension clinic of the Parkland Hospital. Specimens were also collected from a series of rats and dogs in which chronic hypertension had been induced at least 6 months previously by the application of a figure-of-eight ligature to the right kidney and ablation of the left kidney (Grollman, 1944). The average resting blood pressures of the human patients are given in a subsequent table. In the case of the rats, the mean blood pressures as determined daily for periods of several weeks prior to this study ranged between 150 and 200 mms. as determined by the plethysmographic method of Williams, Harrison, and Grollman (1939), In the case of the dogs, the mean arterial blood pressure as determined by direct intra-arterial puncture with a needle connected to a mercury manometer, ranged between 150 and 180 mms.

As controls, urine collections were made in a comparable manner from normotensive men, dogs, and rats and their antidiuretic activity compared with that of the urine from the hypertensives.

In the case of the human urines (Tables 1 and 2), the 24-hour specimens or aliquots of same were finally made up to a volume equivalent to 100 cc. per 24-hours excretion. One cc. of the final extract was injected for every 100 g. of body weight of the test animals. In the case of the dogs (Table 3), the entire volume of urine was concentrated as described in the preceding paper and made up to a final solution of 10 cc. In the case of the rats (Table 4),

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TABLE 1. THE ANTIDIURETIC ACTIVITY OF THE URINE OF HYPERTENSIVE PATIENTS AS BIOASSAYED ON RATS

| Patient | Age | Sex | Blood pressure | Urinary excretion in 24 hours | Chloride excretion in 50% urine | Time for 50% urinary excretion |
|---|---|---|---|---|---|---|
| | | | mms. Hg. | cc. | m.eq. | mins. |
| J.H. | 65 | F | 220/130 | 1200 | 2.1 | 165 |
| A.W. | 45 | F | 150/102 | 1400 | 0.8 | 100 |
| G.R. | 53 | M | 190/110 | 940 | 1.0 | 180 |
| O.E. | 33 | M | 190/130 | 2320 | 1.1 | 180 |
| E.S. | 44 | F | 180/118 | 1700 | 1.1 | 180 |
| J.B. | 48 | M | 190/110 | 1100 | 1.2 | 180 |
| D.V. | 49 | F | 170/110 | 2700 | 1.6 | 180 |
| J.V. | 23 | M | 200/140 | 1330 | 1.7 | 180 |
| E.H. | 45 | F | 154/108 | 1300 | 0.7 | 155 |
| B.H. | 46 | F | 175/140 | 1675 | 0.3 | 130 |
| M.C. | 24 | F | 194/134 | 1350 | 0.9 | 90 |
| S.D. | 37 | F | 198/120 | 1350 | 1.3 | 104 |
| P.S. | 60 | F | 170/100 | 700 | 1.0 | 160 |
| B.S. | 52 | F | 154/104 | 950 | 0.6 | 150 |
| M.E. | 47 | F | 170/110 | 1000 | 1.1 | 180 |

TABLE 2. THE ANTIDIURETIC ACTIVITY OF THE URINE OF NORMOTENSIVE SUBJECTS AS BIOASSAYED ON RATS

| Subject | Age | Sex | Blood pressure | Urinary excretion in 24 hours | Chloride excretion in 50% urine | Time for 50% urinary excretion |
|---|---|---|---|---|---|---|
| | | | mms. Hg. | cc. | m.eq. | mins. |
| M.E. | 28 | M | 110/70 | 800 | 2.0 | 130 |
| J.V. | 26 | M | 112/80 | 1750 | 0.7 | 112 |
| L.T. | 27 | M | 115/80 | 700 | 0.7 | 115 |
| C.B. | 38 | M | 110/80 | 1550 | 1.1 | 92 |
| C.R. | 23 | M | 115/85 | 1100 | 0.6 | 100 |
| L.L. | 26 | M | 115/80 | 1050 | 1.0 | 114 |
| A.G. | 47 | M | 100/70 | 1100 | 1.2 | 116 |
| E.G. | 52 | F | 110/75 | 1210 | 0.8 | 105 |
| A.F. | 36 | F | 108/70 | 1300 | 1.0 | 122 |

TABLE 3. THE ANTIDIURETIC ACTIVITY OF THE URINE OF NORMAL AND HYPERTENSIVE DOGS EXPRESSED AS MILLIUNITS OF POSTERIOR PITUITARY SOLUTION

| Normotensive Controls | | | Hypertensive Dogs | | |
|---|---|---|---|---|---|
| Mean blood pressure | Antidiuretic potency of urine | Amount of antidiuretic principle excreted | Mean blood pressure | Antidiuretic potency of urine | Amount of antidiuretic principle excreted |
| mms. Hg. | milliunits per 100 cc. | milliunits per day | mms. Hg. | milliunits per 100 cc. | milliunits per day |
| 100 | 30 | 60 | 150 | 55 | 660 |
| 110 | 44 | 68 | 160 | 225 | 630 |
| 115 | 117 | 214 | 170 | 296 | 858 |
| 95 | 62 | 76 | 180 | 309 | 572 |
| 100 | 80 | 84 | 180 | 270 | 553 |
| 105 | 70 | 182 | 160 | 118 | 419 |

the entire urinary output was also concentrated but diluted only to 2 cc. The doses administered in Tables 1 and 2, therefore, correspond to 1 per cent of the daily output per 100 g. of body weight of the test rats. In the case of Table 3, the results are given in terms of the daily excretion of the antidiuretic principle. In the case of Table 4, comparable amounts of the urine from normotensive and hypertensive animals were administered.

Controls in which 0.9 per cent saline was administered in doses of 1 cc. per 100 g. of body weight of the experimental rat were always run concurrently with the urinary extracts. The control figures after saline and after

TABLE 4. THE ANTIDIURETIC AND CHLORURETIC ACTIVITIES OF THE URINE OF NORMAL AND HYPERTENSIVE RATS

| Normotensive Controls | | | Hypertensive Rats | | |
|---|---|---|---|---|---|
| Mean blood pressure | Chloride excretion | Time for 50% urinary excretion | Mean blood pressure | Chloride excretion | Time for 50% urinary excretion |
| mms. Hg. | m.eq. per 100 g. | mins. | mms. Hg. | m.eq. per 100 g. | mins. |
| 100 | 0.9 | 80 | 180 | 0.9 | 180 |
| 110 | 0.7 | 95 | 170 | 2.8 | 180 |
| 90 | 1.6 | 75 | 200 | 1.4 | 180 |
| 105 | 0.6 | 90 | 160 | 1.6 | 170 |
| 100 | 2.1 | 80 | 150 | 1.5 | 170 |
| 95 | 1.8 | 80 | 210 | 0.6 | 180 |

known doses of posterior pituitary extract were within the range of the values reported in the preceding paper (Grollman and Woods, 1949).

## RESULTS

The chloruretic and antidiuretic activities obtained from the urine of hypertensive and normotensive humans are given in Tables 1 and 2, respectively. The tables show the absence of chloruretic activity in urine and the presence of an increased antidiuretic activity of the urine of hypertensive patients as compared to normotensive individuals. Of the 15 patients cited in Table 1, the antidiuretic activity is increased in 11 cases and is in the normal range in only 4. No difference was noted in the antidiuretic action of the first 6 subjects cited in Table 2 who were non-smokers as compared to the last 3 subjects who were heavy smokers. Any stimulating effect of nicotine or of smoking on the posterior pituitary gland (Burn, Truelove, and Burn, 1945) is thus not reflected in an increased excretion of the antidiuretic principle of the urine.

In Table 3 are given the results obtained on 5 hypertensive dogs as compared to a similar number of normotensive animals. Although the concentration of antidiuretic potency of the urine from the hypertensive dogs was within the range of normal in 2 cases, the total amount of the antidiuretic principle excreted in 24 hours was increased in every case, ranging from 419 to 858 milliunits per day in the hypertensives as compared to only 60 to 214 in the normotensive

dogs. There is thus a definite increase in the amount of antidiuretic principle excreted in the urine daily in the hypertensive dog. A similar state of affairs is also observed in the case of chronic hypertension in the rat. As shown in Table 4, the urine of the hypertensive animals exerts in every case a marked antidiuretic activity while urine from normotensive animals is devoid of such action.

## DISCUSSION

The present results demonstrate an increased excretion of antidiuretic principle in the urine of hypertensive patients and of dogs and rats with induced experimental renal hypertension. Hypertensive cardiovascular disease must thus be added to cirrhosis of the liver with ascites (Ralli, *et al.*, 1943), eclampsia (Teel and Reid, 1939), and acute glomerular nephritis (Robinson and Farr, 1940) in which an increased rate of excretion of this principle also has been observed. The significance of the presence of an increased amount of the antidiuretic principle in the urine in these conditions is still a matter for speculation. Griffith and his coworkers (Griffith, *et al.*, 1941; Pendergrass, *et al.*, 1947) have suggested that the posterior pituitary gland is hyperactive in some cases of hypertension and have recommended measures designed to reduce its activity. However, there is little to support the contention of a posterior-pituitary origin of hypertension (Grollman, 1947).

All of the clinical conditions cited above in which an increased excretion of antidiuretic principle is observed have in common the tendency for the retention of salt and water. It is questionable, however, if the increased antidiuretic action of the urine represents an increased rate of secretion of the antidiuretic hormone which is responsible for the altered salt and water metabolism, or merely a secondary response to the primary disturbance in kidney or liver which is responsible for the altered salt and water metabolism. The possibility also remains that the appearance of an increased amount of antidiuretic principle in the urine does not reflect any increased activity of the posterior lobe of the pituitary but rather a decreased rate of destruction of the hormone. In fact, the generally assumed role of the posterior pituitary in regulating water metabolism has been questioned (Newton and Smirk, 1934). Other evidence indicates a possible extra-pituitary origin of the antidiuretic principle found in urine (Walker, 1939).

The fact that the antidiuretic activity of the urine is increased in both clinical hypertension of man as well as in experimental renal hypertension in animals is further evidence for the probable identity of these disorders (Grollman, 1947). Frankel and Wakerlin (1943) were unable to detect an increased excretion of the antidiuretic principle in hypertensive dogs. Their failure to do so may be attributed to the methods used by these workers. The use of the procedure outlined

in the preceding paper, however, as shown in Table 3, gives ample evidence that the dog is not unique but resembles man and the rat in the excretion of an increased amount of the antidiuretic principle in the urine when rendered hypertensive.

## SUMMARY

The antidiuretic activity of the urine of normotensive men, dogs, and rats has been compared with that observed in patients with hypertension and dogs and rats with experimental renal hypertension. In 15 human hypertensives, the excretion of the antidiuretic principle was increased in 11 and within the normal range in the remaining 4. In the case of 6 hypertensive dogs, the daily excretion of the antidiuretic principle varied from 419 to 858 milliunits as compared to 60 to 214 milliunits in an equal group of normotensive animals. The urine of hypertensive rats is also strongly antidiuretic while that of normotensives exerts no such action when concentrated to the same degree. The possible significance of these observations is discussed.

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# THE INFLUENCE OF ASCORBIC ACID ON THE ADRENAL WEIGHT DURING EXPOSURE TO COLD

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It is well known that any type of stress produces an increase in weight of the adrenals, and it is believed that the weight response in such conditions is an aspect of the functional adaptation of the adrenals to the increased requirement for cortical hormones by the organism (Selye, 1937) (Ingle, 1938, 1939); in other words, that the gain in adrenal weight, under conditions of stress, is due to an hypertrophy of the cortex with a corresponding increase in adrenal cortical hormones production, and consequently an increased resistance towards the damaging agent. It has also been shown by many authors that the enlargement of the adrenal cortex can be prevented (Ingle, 1938) (Selye, 1940) or even more that the gland may become atrophied (Selye and Dosne, 1942) when the animals under stress are treated with sufficiently high doses of adrenal cortical hormones; moreover, such a simple chemical substance as ammonium chloride has been reported to inhibit slightly the adrenal enlargement normally induced by anterior pituitary extract (Hall and Selye, 1945).

On the other hand, it has been reported quite often in recent years that a relation of some sort—still obscure—seems to exist between the ascorbic acid level of the adrenal cortex and the hormonal activity of the latter: Giroud, Santa and Martinet (1940), Giroud, Santa, Martinet and Bellon (1940), Giroud, Martinet and Bellon (1941), Giroud and Martinet (1941) have observed that not only does ascorbic acid favour the production of adrenal cortical hormones but also improves its utilization; another aspect of the same problem has been worked out by Long and his collaborators (Sayers, Sayers, Fry, White and Long, 1944), (Harkins and Long, 1945), (Long, 1947) who have found a decrease in ascorbic acid and cholesterol content of the adrenals after injection of adrenotrophic hormone or exposure to all sorts of stress including cold: those changes are seen to occur a few hours after injury; Dugal and Thérien (1947) have reported an increase in adrenal ascorbic acid for guinea-pigs and rabbits, and in other tissues for rats and guinea-pigs, during long exposures to a cold environment, when those animals get adapted to low temperatures;

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if not, the contrary occurs, namely a decrease in adrenal ascorbic acid before the animals die; the same authors also found a direct relation between ascorbic acid content of the adrenals and adaptability to cold; subsequently, Ludewig and Chanutin (1947) have also found an increase in adrenal ascorbic acid (following the initial decrease) after injury (not including exposure to cold), and Thérien and Dugal (1949) following the urinary excretion of ascorbic acid during exposure to cold were able to confirm indirectly the above findings of Long (1947), and their own (1947).

In view of all those relations between stress, on the one hand, and adrenal enlargement, adrenal ascorbic acid changes, adrenal ascorbic acid level and acclimatization, on the other, and also in view of the fact that large intake of ascorbic acid increases resistance to haemorrhagic shock (Stewart, Learmonth and Pollock, 1941) (Dosne de Pasqualini, 1946) and to cold (Dugal and Thérien, 1947), it was only natural to wonder what would be the effect of relatively large doses of ascorbic acid on the adrenal hypertrophy normally observed during short or long (Emery, Emery and Schwabe, 1940) exposure to cold.

## EXPERIMENTAL

Three main experiments were conducted using white rats in the first ones and guinea-pigs in the last one.

1) Short exposure of white rats to cold.

A—*Technique.* In the first experiment, in which we wanted to study the effect of ascorbic acid on adrenal weight during a short and acute exposure to cold, 140 male white rats weighing around 200 grams were divided into four groups: two of them were exposed to a temperature of −1°C for 72 hours, the experimental group receiving daily, by intraperitoneal injections, 150 mgm. of ascorbic acid in the form of sodium ascorbate,* at the rate of three injections daily of 0.5 cc. each time, each injection being equivalent to 50 mgm. of ascorbic acid; the control group received daily an equivalent volume of a sodium chloride solution (0.9%) equally divided into 3 intraperitoneal injections. The animals were kept in individual cages and fed "Purina Fox Chow" ad libitum; the food consumption was measured and found to be the same for both groups.

Drinking water was also given ad libitum and replaced as often as necessary to avoid freezing. Two corresponding groups were kept at room temperature. All animals were sacrificed at the end of the three day period.

B—*Results.* a) A most striking and unexpected result was found: *the increase in adrenal weight,* normally seen in rats submitted to such a cold temperature as we have used and for such a lapse of time, *has been prevented by ascorbic acid:* this is clearly shown in Table 1, where

* "Redoxon forte" from Hoffman-Laroche, Montreal.

TABLE 1. INFLUENCE OF ASCORBIC ACID ON ADRENAL WEIGHT. SHORT EXPOSURE (72 HOURS) TO A TEMPERATURE OF −1°C

| | Room Temperature | | | | Cold Room | | | | % Increase | t |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number of animals | Weight of animals | | Adrenal weight mgm. | Number of animals | Weight of animals | | Adrenal weight mgm. | | |
| | | Initial | Final | | | Initial | Final | | | |
| Group I* | 24 | 200.4 | 204.6 | 33.45 ±1.36‡ | 33 | 201.3 | 190.6 | 33.70 ±1.12 | 0.74 | 0.14 |
| Group II† | 25 | 199.9 | 204.2 | 32.47 ±0.97 | 33 | 202.1 | 192.6 | 36.09 ±0.99 | *11.10* | *2.64* |

* Received 150 mgm./day of ascorbic acid.
† Received 0.9% NaCl.
‡ Standard error.

the adrenals of the group receiving ascorbic acid have an average weight of 33.45 mgm. at room temperature and 33.70 mgm. at −1°C; the increase is less than 1% and is not significant (t = 0.14). On the other hand, the untreated group shows the typical increase in adrenal weight: the average values for room temperature and −1°C are respectively 32.47 mgm. and 36.09 mgm., representing an increase of 11.1% in the adrenal weight of the animals submitted to cold. This last result is statistically significant (t = 2.64).

b) Controls of each group (receiving ascorbic acid or sodium chloride) at room temperature show no significant difference in adrenal weight.

c) Survival of the ascorbic acid group in the cold room is definitely better than that of the controls submitted to the same temperature, if the experiment lasts at least a week, in the same conditions of temperature and with animals of the same weight as above (Table 2).

TABLE 2. INFLUENCE OF ASCORBIC ACID ON SURVIVAL OF RATS SUBMITTED DURING 7 DAYS TO A TEMPERATURE OF −1°C

| | Number of animals | Number of dead | Survival |
|---|---|---|---|
| Group receiving 150 mgm./day of ascorbic acid | 12 | 3 | 75% |
| Controls receiving sodium chloride | 12 | 12 | 0% |

It must be emphasized that after 3 days of exposure to cold in the conditions described, the difference in resistance between the two groups *cannot be appreciated* from the difference in survival, both groups *seeming* at that time equally resistant. Incidentally, it may be said here that doubtful results about the beneficial effect of ascorbic acid for resistance to cold, like the ones of Grab and Lang (1946) may be due to the use of only one criterion, the survival, during a too short period of time.

d) It is clear, from Table 3, that the control group in the cold room shows an increase in kidney weight (as compared to room temperature) but not the group receiving ascorbic acid.

2) Long exposure of white rats to cold.

A—*Technique.* In the second experiment which was performed on

TABLE 3. INFLUENCE OF ASCORBIC ACID ON KIDNEY WEIGHT. SHORT EXPOSURE (72 HOURS) TO A TEMPERATURE OF −1°C

| | Room Temperature | | | | Cold Room | | | | % Increase | t |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number of animals | Weight of animals Initial | Weight of animals Final | Kidney weight gm. | Number of animals | Weight of animals Initial | Weight of animals Final | Kidney weight gm. | | |
| Group receiving ascorbic acid | 25 | 200.4 | 204.6 | 2.141 ±0.05 | 32 | 201.3 | 190.6 | 2.057 ±0.05 | −3.92 | 1.1 |
| Control group receiving NaCl | 25 | 199.9 | 204.2 | 2.076 ±*0.05* | 32 | 202.1 | 192.6 | 2.225 ±*0.05* | 7.17 | *2.13* |
| Difference | | | | 3.13% (t = 0.95) | | | | 8.1% (*t = 2.37*) | | |

130 male white rats, weighing around 250 grams, two groups were exposed to a relatively mild cold of 4°C for a period of 244 days. One of the two groups received daily, by mouth, 25 mgm. of ascorbic acid, and the control group, tap water. Corresponding groups were also kept at room temperature. All other conditions were similar to those described for the first experiment.

B—*Results*. The results are the same as in the first experiment, but still more obvious.

a) The same phenomenon, that *ascorbic acid prevents the hypertrophy of the adrenals*, during exposure to cold, is still more visible here than it was for a short exposure to cold (Table 4). The average

TABLE 4. INFLUENCE OF ASCORBIC ACID ON ADRENAL WEIGHT. LONG EXPOSURE (244 DAYS) TO A TEMPERATURE OF 4°C

| | Room Temperature | | | | Cold Room | | | | % Increase | t |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number of animals | Weight of animals Initial | Weight of animals Final | Adrenal weight mgm. | Number of animals | Weight of animals Initial | Weight of animals Final | Adrenal weight mgm. | | |
| Group receiving ascorbic acid | 24 | 269 | 364 | 36.90 ±1.75 | 28 | 256.4 | 305.5 | 37.90 ±1.15 | 2.7 | 0.48 |
| Control group (tap water) | 22 | 270 | 360 | 36.65 ±*1.89* | 18 | 270.6 | 301.3 | 44.88 ±*2.98* | 22.4 | *2.85* |
| Difference | | | | nil | | | | 18.4% (*t = 2.18*) | | |

adrenal weight for the group receiving ascorbic acid is about the same at room temperature or in the cold room. The control group, in the cold room, shows an increase in adrenal weight of over 20% (as compared with animals of the same age kept at room temperature).

b) Survival in the cold room is clearly favoured by ascorbic acid (see Table 5).

TABLE 5. INFLUENCE OF ASCORBIC ACID ON SURVIVAL OF RATS SUBMITTED 244 DAYS TO A TEMPERATURE OF 4°C.

| | Number of animals | Number of dead | Survival |
|---|---|---|---|
| Group receiving ascorbic acid | 35 | 7 | 80.0% |
| Group receiving tap water | 35 | 17 | 51.4% |

As a matter of fact, the ascorbic acid group submitted to cold did just as well as the controls of each group kept at room temperature.

c) Table 4 also shows that the gain in weight in the cold room is greater for the ascorbic acid group than for the control group, the per cent increases in average weight being respectively 19.1 and 11.2.

3) Long exposure of guinea-pigs to cold.

The previous results obtained on rats raised the question as to what effects ascorbic acid would have on adrenal weight of guinea-pigs, unable to synthesize that vitamin, during relatively long exposure to cold.

A—*Technique.* Two experiments were performed differing by the quantity of ascorbic acid administered daily, the initial weights of the animals (male guinea-pigs) when first exposed to cold, the temperature of the cold room and the duration of exposure. All groups received their ascorbic acid by mouth, Group II, 25 mgm. daily, Group VI, 10 mgm., and Group IX, 2 mgm. The animals were placed

TABLE 6. INFLUENCE OF ASCORBIC ACID ON ADRENAL WEIGHT. GUINEA-PIGS EXPOSED TO COLD FOR 90 DAYS*

| | Room Temperature | | | | | Cold Room | | | | | % Increase | t |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Number of animals | Body weight Initial | Body weight Final | Average ascorbic acid mgm./gm. adrenals | Adrenal weight mgm. | Number of animals | Body weight Initial | Body weight Final | Average ascorbic acid mgm./gm. adrenals | Adrenal weight mgm. | | |
| Group II | 25 | 375.0 | 626.4 | 0.742 | 375.0 ±24.86 | 26 | 449.4 | 486.8 | 0.857 | 409.2 ±16.97 | 9.1 | 1.12 |
| Group IX | 25 | 391.8 | 654 | 0.182 | 369.7 ±*20.32* | 23 | 447.7 | 472.3 | 0.167 | 475.5 ±*18.92* | 28.6 | *3.81* |
| Difference | | | | | 1.4% (t=0.16) | | | | | 16.2% (*t=2.62*) | | |

* (−8°C for 12 hours and +8°C for 12 hours every day.)

in individual cages. The food ("Purina Rabbit-Chow" was given ad libitum, measured and found to be consumed in equal quantities by the different groups exposed to the same temperature, but the consumption was greater in the cold room than at normal temperature. Drinking water was also given ad libitum.

B—*Results.* a) The results presented in Table 6 show exactly the same phenomenon for guinea-pigs as the ones described above for white rats, namely that the hypertrophy of the adrenals is significantly smaller in the group receiving more ascorbic acid. In fact, the group receiving 25 mgm. daily (per os) of ascorbic acid shows an adrenal enlargement of 9.1% which is statistically insignificant while the adrenal weight of the other group which received only 2 mgm. a day of Vitamin C is 28.6% greater in the cold room than at room temperature, and the result is highly significant (t = 3.81). The difference between the adrenal weights of each group is very small and not significant at room temperature, but significant (16.2%; t = 2.62 in the cold room.

The average ascorbic acid concentration of the adrenals is also shown. It is—as expected—larger in group II than in group IX; besides, if one compares the concentrations for ascorbic acid for the same group at room temperature and in the cold room respectively, one finds that for group II, more ascorbic acid is retained in the adrenals of the subjects exposed to cold: the difference is significant and is in line with our previous results (Dugal and Thérien, 1947). The difference for group IX, in the same conditions, is not significant.

b) One other experiment is summarized in Table 7: the groups

TABLE 7. INFLUENCE OF ASCORBIC ACID ON THE ADRENAL WEIGHT OF GUINEA-PIGS EXPOSED TO 2°C FOR 40 DAYS

| | GROUP VI | | | | GROUP IX | | | | Difference in relative adrenal weight | t |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number of animals | Body weight Initial | Body weight Final | Adrenal wt./Body wt. mgm./gm. | Number of animals | Body weight Initial | Body weight Final | Adrenal wt./Body wt. mgm./gm. | | |
| Cold Room | 24 | 437.4 | 506.8 | 0.6489 | 25 | 425.3 | 467.4 | 0.7628 | 17.5% | 2.32 |
| Room Temperature | 25 | 449.1 | 654.8 | 0.4760 | 23 | 457.2 | 659.0 | 0.4549 | 4.6% | 0.92 |

used received respectively 10 mgm. (Group VI) and 2 mgm. (Group IX) a day of ascorbic acid by mouth, the experiment lasted 40 days instead of 90, and the temperature was maintained constantly at 2°C throughout the experiment. The results presented are for adrenal weight as related to the body weight: this seemed to be the only logical procedure to follow, since all groups gained weight—even in the cold room—but the gain in weight was highly unequal for the different groups (in the cold room, the group receiving more ascorbic acid gained more weight).

Here again, it is obvious that ascorbic acid has an influence on adrenal weight at low temperature (the more ascorbic acid being taken, the smaller being the adrenal weight), but not at room temperature.

c) Oedema of the penis, one of the symptoms of non adaptation to cold (Leblond and Dugal, 1943) occurs more frequently in groups of guinea pigs receiving less ascorbic acid (ratio = 6:1 for the two experiments).

## SUMMARY AND CONCLUSIONS

*The typical enlargement of the adrenals* under the influence of stress, observed many times before by other workers, and by ourselves for our controls in this actual series of experiments where cold was the damaging agent, *is completely prevented,* in rats and guinea-pigs exposed to cold if *they receive large doses of ascorbic acid.*

Surprising as it may seem, it is the very animals which have the smaller adrenals (those which receive the large doses of ascorbic acid) which are—and by far—the more resistant to cold.

Those experiments show that ascorbic acid seems to play a com-

pensatory role somewhat similar to the one of adrenal cortical hormones. The normal hypertrophy is prevented—although there is no atrophy—but, at the same time, the resistance to cold is increased. All those results, bringing new fundamental facts about the beneficial physiological effects of ascorbic acid in the cold, confirm our previous findings on the role of ascorbic acid for resistance and acclimatization to cold.

It is the first time, to our knowledge, that a substance other than a hormone, a substance which is *called* a Vitamin, is reported to prevent entirely, at least in the conditions that we have been using, the hypertrophy of the adrenals in a case of stress.

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# THE ACTION OF TESTOSTERONE ON THE ADRENAL CORTEX OF THE HYPOPHYSECTOMIZED, PREPUBERALLY CASTRATED MALE MOUSE[1]

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THE ADRENAL cortex of the adult male and the parous female mouse consists of a connective tissue capsule surrounding the whole gland, an outer zona glomerulosa, a zona fasciculata and generally but not invariably a zona reticularis and a medullary connective tissue capsule. In young mice and in virgin females a further zone is recognizable, namely, the X zone and this shows variations with sex and age. In those mice with an X zone a medullary connective tissue capsule is not present and a zona reticularis is not well-developed or is absent (Waring, 1935; Jones, 1948a). For convenience of description the zona glomerulosa and the zona fasciculata (and the zona reticularis) are together termed the "permanent" cortex. In contradistinction to this term the X zone has been referred to as the "transitory" cortex (Whitehead, 1933) but the former term will be employed in this paper as it is now the name in common usage.

The variations of the X zone in the female mouse adrenal are not dealt with in this paper. In the male, however, the X zone disappears regularly at maturity, the degeneration usually being completed by 40 days (Howard, 1927; Deanesly, 1928; Whitehead, 1933; Waring, 1935 and others). Castration of the immature male mouse, preferably at about 21–25 days of age allows the X zone to remain and to increase in size. Such prepuberally castrated male mice have been studied by Deanesly and Parkes (1937), who showed that injections of androgens caused the disappearance of the X zone so that the adrenals then assumed the histological appearance characteristic of those of normal adult mice. Suppression of the X zone by testosterone in both male and female mice has also been shown (see Howard, 1940, for references). It is unknown whether or not this destructive action of androgens is a direct one on the X zone or mediated through the pituitary. The results of an experiment designed to elucidate this problem by the use of hypophysectomized, prepuberally castrated male mice

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are given here. Included in this paper are also the results of short term treatment with testosterone on the permanent cortex of the adrenals of the mice in the different experimental categories.

## MATERIAL AND METHODS

Male mice of the "Swiss albino" strain were used. Twenty-eight were castrated at 21 days of age and left for 12 days (during which time the adrenal X zone persists and increases in size). At 33 days of age the mice were divided into the four groups delineated below. All the mice were killed at 42 days of age.

I. Six untreated castrated mice.
II. Six castrated mice injected subcutaneously once daily with 1 mg. of testosterone propionate in sesame oil.[3] The injections were started at 34 days of age, repeated for seven days, the animals being killed 24 hours after the last injection.
III. Six castrated mice were hypophysectomized and killed nine days later.
IV. Ten castrated mice were hypophysectomized. Subcutaneous injections, once daily, with 1 mg. of testosterone propionate in sesame oil[3] were given for 7 days, starting 24 hours after operation. The animals were killed 24 hours after the last injection.

The average of the body and the seminal vesicle weights of these four groups of mice are given in Table 1. The testosterone treated animals were livelier

TABLE 1. AVERAGE BODY AND SEMINAL VESICLE WEIGHT OF THE FOUR GROUPS OF MICE, AT DEATH

| Group | Number in Group | Treatment* | Body Weight in gms. | Weight of seminal vesicles in mgs. |
|---|---|---|---|---|
| I | 6 | Castrated | 18.8 | 2.4 |
| II | 6 | Castrated; testosterone injected | 21.7 | 45.1 |
| III | 6 | Castrated; hypophysectomized | 13.8 | 2.2 |
| IV | 10 | Castrated; hypophysectomized, testosterone injected | 15.8 | 28.5 |

* For details, see text.

and "sleeker" than the controls. However, the actual body weight differences between Groups I and II and between Groups III and IV are of doubtful significance. Seminal vesicle weights in Groups II and IV as compared to those of Groups I and III indicate that the testosterone was biologically active.

The operation of hypophysectomy was performed by the parapharyngeal approach. Confirmation of the completeness of hypophysectomy was made in the case of ten animals (5 from Group III and 5 from Group IV) by exami-

[3] "Perandren," Ciba Pharmaceutical Products, Inc., supplied through the courtesy of Dr. Houghton.

nation of sections made of the appropriate area. In the remainder, the success of the operation was judged by examination of the sella under the binocular microscope. It was found that this macroscopic examination is a complete confirmation once the individual technique is established. Hypophysectomy is here interpreted as the absence of the anterior and intermediate lobes (see Newton and Richardson, 1940, for discussion).

The mice, after castration and until the termination of the experiment, were kept on a high caloric synthetic diet (Shaw, 1947, gives the composition). It has been found that the chance of survival of hypophysectomized rats is greatly enhanced by the use of high caloric diets (Shaw and Greep, unpublished), and the results with mice used in this experiment and in other series confirm this (Jones, 1948b and unpublished).

The mice were killed with chloroform. The right adrenal was fixed in Bouin's fluid, embedded in paraffin, cut at 7$\mu$ and stained with Harris' haemotoxylin and eosin, or Heidenhain's Azan. The left adrenal was fixed in 10% formalin for at least 48 hours, washed in running water for an hour and sectioned on the freezing microtome at 15$\mu$. Six sections from each gland were taken and treated as follows: (1) stained with Sudan black; (2) stained with Sudan IV; (3) stained with Sudan IV and counterstained with haemotoxylin; (4) treated with the Schiff reagent; (5) mounted unstained in glycerine and viewed with the polarizing and with the fluorescent microscopes; and (6) left in acetone at room temperature for half an hour and used as a control section for (5). These methods used on the left adrenal are discussed by Dempsey and Wislocki (1946) and have been employed extensively (see Deane and Greep, 1946; Greep and Deane, 1947; Dempsey, Bunting and Wislocki, 1947). In this paper, however, substances which react positively for all these tests are not regarded as necessarily indicating the presence of ketosteroids. Nevertheless criticism (Albert and Leblond, 1946; Yoffey and Baxter, 1947) which denies this specificity overlooks the extreme usefulness of the methods. The minimum evaluation of the histochemical tests employed here is that Sudan stains expose lipids, the Schiff reagent reacts with the aldehyde group and perhaps identifies in tissue sections the "plasmalogens" (Thannhauser and Schmitt, 1946), that examination with the polarizing microscope reveals anisotropic substances appearing after formalin fixation and that with the fluorescent microscope the presence and distribution of fluorescent substances are shown up in the adrenal cortex, and that these substances are acetone soluble.

Nevertheless, irrespective of any claim for specificity for any type of chemical compound, it is probable that the substances these tests reveal are fairly directly related to and fluctuate in accordance with the hormone (or hormones) so abundantly produced by the normal gland. Furthermore the reactivity with these tests is dependent on the endocrine status of the gland at the time and can be correlated with the state of activity of the adrenal cortex. For instance, good pointers to secretory state are given by consideration of the size and distribution of sudanophilic droplets within the cells, the predominance of the type of birefringence, whether coarse or fine, the intensity of the color after the Schiff reagent, and the particular areas which contain autofluorescent material. It is to be expected that tissues positive to some of these tests are scattered throughout the body. For example, any tissue with a molecular orientation is double refracting, fats in general are in

the class "lipids" and are sudanophilic, and Schiff positive substances are widespread throughout the body. However, the significant fact must not be overlooked that only the known steroid producing organs in their normal condition give positive results with all five of these tests. In brief, as regards the adrenal gland it is certain that these tests expose cortical changes in a more sensitive manner than the routine methods. Furthermore, these changes can be interpreted to throw light on the state of its activity.

## RESULTS

1. The adrenal of the untreated prepuberally castrated male shows a well-developed X zone (Plate I, fig. 1). In the haemotoxylin and eosin preparations the zone is compact with the cytoplasm of the cells densely eosinophilic, the nuclei large and basophilic. The zone is unmistakable and has been described frequently after routine preparations as has the permanent cortex (Howard, 1927; Deanesly, 1928; Whitehead, 1933). After the lipid histochemical methods, the X zone is not sudanophilic, not Schiff-positive, contains no birefringent material (Plate II, figs. 5 and 9; Plate III, fig. 13) and is not autofluoresecent. The zona glomerulosa and zona fasciculata are positive for these tests; the Sudan and the Schiff reagents give strong colors (figs. 5 and 9) the reaction being within the droplets and autofluorescence being shown by both zones. The birefringent particles are both coarse and fine, scattered throughout the two zones (fig. 13). The acetone extracted control section is not autofluorescent or birefringent.

2. In the adrenal of the prepuberally castrated male injected with testosterone the X zone has disappeared and a medullary connective tissue capsule has formed (Plate I, fig. 2). The adrenal after the histochemical methods gives much the same picture as for the control (1 above). The Sudan and Schiff reactions, however, give less strong colors and there are fewer fine birefringent particles scattered in the cortex (Plate II, figs. 6 and 10; Plate III, fig. 14). A few big sudanophilic droplets lie in the juxtamedullary cells (fig. 6); these are Schiff

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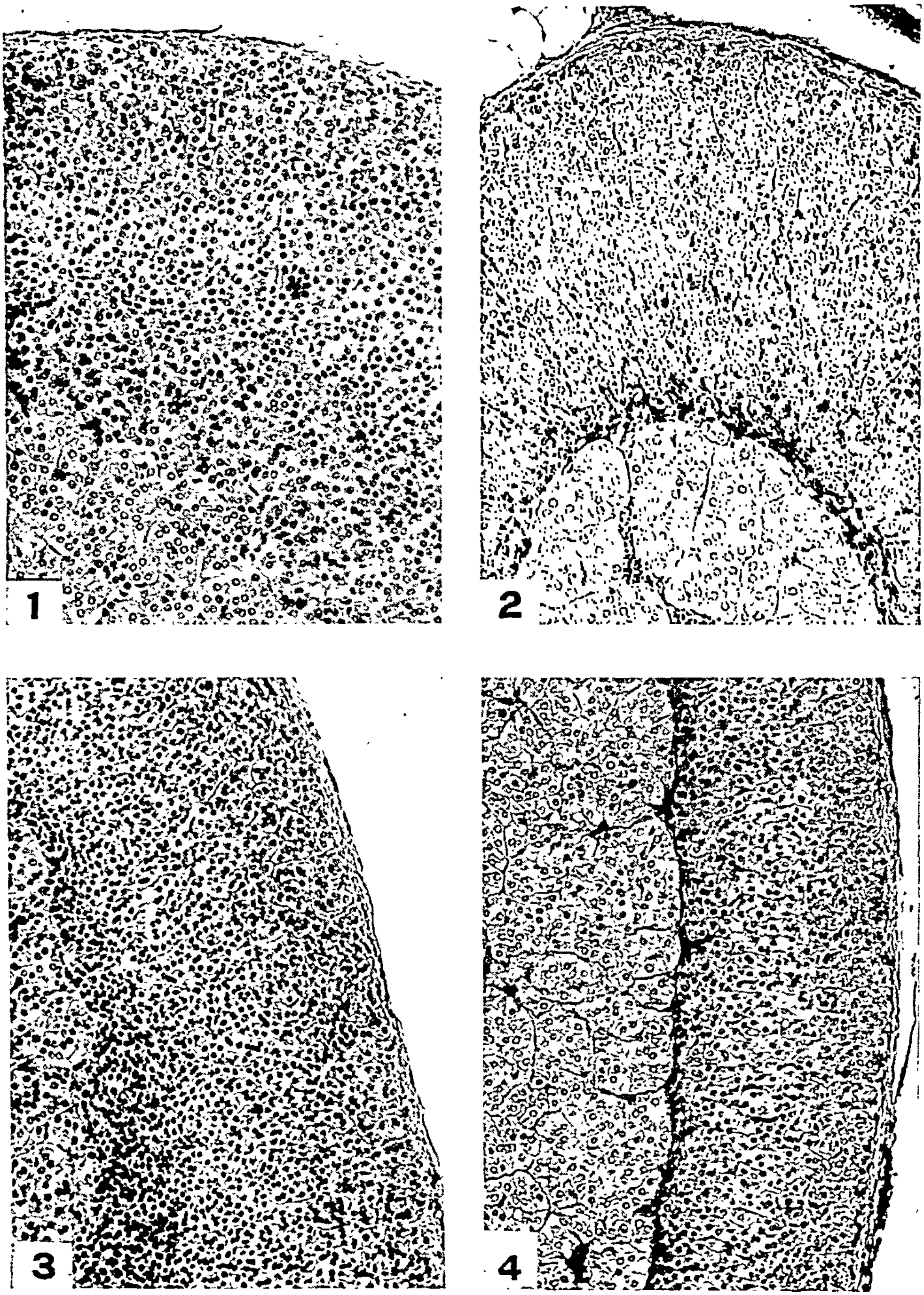
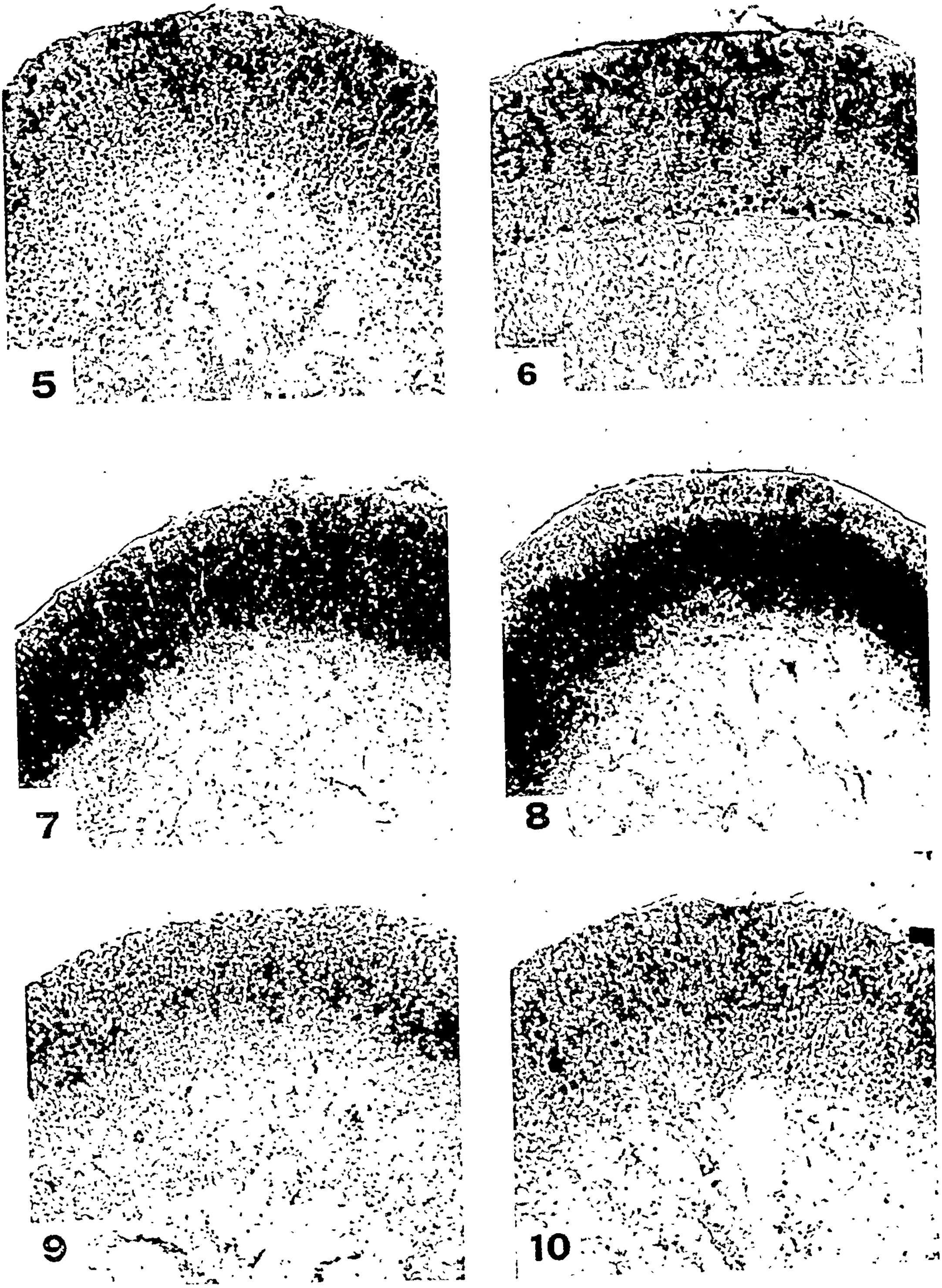
### Description of Plates

Plates I, II and III are section of adrenals from male mice, castrated at 21 days of age and killed at 42 days of age. Other experimental treatment in the different categories is indicated in each figure legend, the precise details being given in the text.

Plate I. Figs. 1 and 3 Harris' haemotoxylin and eosin; figs. 2 and 4 Heidenhain's Azan. ×190.

1. Castrated mouse. Juxtamedullary X zone well-developed.
2. Castrated mouse, injected with 1 mg. testosterone propionate daily for 7 days. The X zone has disappeared and a medullary connective tissue capsule formed.
3. Castrated mouse, hypophysectomized for 9 days. The X zone has condensed, the nuclei pycnotic; the zona fasciculata shows atrophy, the zona glomerulosa is little affected by the operation.
4. Castrated mouse, hypophysectomized for 9 days, injected with 1 mg. per day of testosterone propionate for 7 days. The X zone has disappeared, a medullary connective tissue capsule has formed; the zona fasciculata is atrophic, the zona glomerulosa is little affected.

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negative (fig. 10) and do not contain birefringent particles (fig.14). The "permanent" cortex is autoflourescent. The acetone extracted control section is not autofluorescent or birefringent.

3. In the adrenal of the hypophysectomized, prepuberally castrated male, after the routine preparation, the zona fasciculata shows the changes characteristically consequent on this operation, with shrinkage of cell cytoplasm, and the pycnosis of nuclei. The zona glomerulosa shows little or no change. The X zone is still distinguishable; it is less eosinophilic, somewhat condensed, with the nuclei pycnotic (Plate I, fig. 3). After the histochemical methods, the sudanophilic droplets in the zona glomerulosa are coarser and less numerous than in the controls (Plate II, fig. 7), they are less Schiff-positive (Plate III, fig. 11), less autofluorescent, and the birefringent particles are coarse and few in number (Plate III, fig. 15). The zona fasciculata is intensely sudanophilic, with the droplets large and tending to coalesce, the color after Schiff is intense; the birefringent particles are coarse for the most part and show a tendency to clump (figs. 7, 11 and 15); autofluorescence is slight to absent. The acetone extracted control section is not autofluorescent or birefringent.

4. In the adrenal of the hypophysectomized prepuberally castrated male injected with testosterone, the X zone has disappeared and a medullary connective tissue capsule formed (Plate I, fig. 4). The routine preparation show the zona glomerulosa and the zona fasciculata in the condition noted for 3 above—the uninjected hypophysectomized animal. The histochemical tests on the left adrenal give constant differences, however. The zona glomerulosa has fewer sudanophilic droplets (Plate II, fig. 8); it is only faintly Schiff-positive or not at all (Plate III, fig. 12); to find birefringent particles in it is rare and these when present are coarse (Plate III, fig. 16); it is not autofluorescent. The zona fasciculata is intensely sudanophilic (fig.

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Plate II. Magnification ×90. Adrenal sections at 15$\mu$ made on the freezing microtome.

5. Castrated mouse, adrenal after Sudan IV. The zona glomerulosa and zona fasciculata have taken the Sudan stain, the X zone lying between the latter zone and the medulla (at the bottom of the photograph) has not.

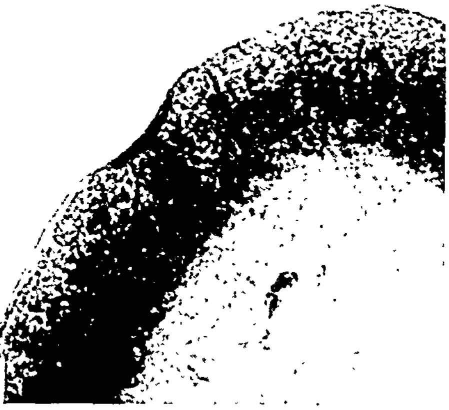
6. Castrated mouse injected with testosterone for 7 days, adrenal after Sudan IV. The Sudan droplets are somewhat sparser and more irregularly distributed in the zona glomerulosa and zona fasciculata. The X zone has been destroyed. A few small very sudanophilic areas lie against the medullary connective tissue capsule.

7. Castrated mouse hypophysectomized for 9 days, adrenal after Sudan IV. The zona fasciculata is densely sudanophilic with coarse droplets; there is rather less sudanophilic material in the zona glomerulosa but the amount present is densely stained.

8. Castrated mouse hypophysectomized for 9 days, injected with testosterone. Adrenal after Sudan IV. The zona fasciculata is densely sudanophilic, the droplets are coarse. The zona glomerulosa is sparsely sudanophilic and can be clearly demarcated from the zona fasciculata.

9. Castrated mouse, adrenal after the Schiff reagent. The zona glomerulosa and zona fasciculata are Schiff positive, the X zone is Schiff negative.

10. Castrated mouse injected with testosterone, adrenal after the Schiff reagent. Both the zona glomerulosa and zona fasciculata are Schiff positive, the color is less intense than that of the control, fig. 9.



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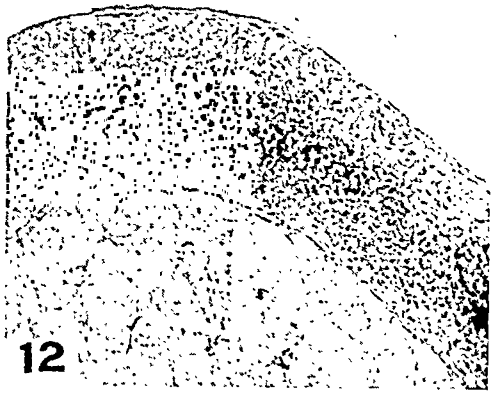

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8), weakly Schiff positive (fig. 12), and full of coarse birefringent particles, largely clumped together (fig. 16). It is faintly autofluorescent or not at all. The acetone extracted control section is not autofluorescent or birefringent.

## DISCUSSION

The persistent adrenal X zone of the prepuberally castrated male mouse is rapidly destroyed by the injection of testosterone and a medullary connective tissue capsule forms. In the hypophysectomized prepuberally castrated male the X zone becomes atrophic but persists as a condensed layer (X zone remnants can still be recognized up to 40 days after operation. (Jones, 1948b and unpublished). This X zone is swept away by exogenous testosterone and a medullary connective tissue capsule appears. It would seem that the action of testosterone on the X zone is a direct one, in the absence of the X zone maintaining principle of the pituitary. This latter has been shown (Jones, 1948c) to be a pituitary gonadotropin, possibly 'LH'. We envisage the mechanism of X zona destruction in the normal male mouse then to be as follows—the androgens produced concomitant with maturity reduce the secretion of pituitary 'LH' (Greep, Van Dyke and Chow, 1942) until a balance is formed and at the same time the androgens act directly on the X zone to destroy it, this destructive action being aided perhaps by the lessening secretion of 'LH'—the X zone maintaining factor.

The function of the X zone is not known. It has frequently been referred to as the "androgenic" zone and assigned the function of androgen secretion. There is no experimental evidence for this view (Gersh and Grollman, 1939a and b; McPhail and Read, 1942; and Howard, 1946). It is significant that the well developed X zone is

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PLATE III. Figs. 11, 12, ×90. Figs. 13 to 16, ×120. Adrenal sections at 15μ made on the freezing microtome.

11. Castrated hypophysectomized mouse, adrenal after Schiff reaction. Positive reaction in permanent cortex, the X zone negative. The zona glomerulosa shows less color than the zona fasciculata, which takes the stain intensely.

12. Castrated hypophysectomized mouse, injected with testosterone, adrenal after Schiff reaction. Little reaction in the zona glomerulosa, positive in the zona fasciculata but weakly so. No X zone.

13. Castrated mouse. Plain section of adrenal mounted in glycerine taken with the polarizing microscope. The permanent cortex has scattered in it a mixture of coarse and fine birefringent particles. The X zone does not show as it contains no birefringent material.

14. Castrated mouse injected with testosterone. Plain section of adrenal mounted in glycerine taken with the polarizing microscope. The cortex contains a mixture of fine and coarse birefringent particles but these are sparse. The apparent wider cortex is not significant, merely being the chosen area.

15. Castrated hypophysectomized mouse. Plain section of adrenal mounted in glycerine taken with a polarizing microscope. The zona glomerulosa shows a few scattered, coarse birefringent particles. The zona fasciculata shows a clumping of this material.

16. Castrated hypophysectomized mouse injected with testosterone. Plain section of adrenal mounted in glycerine taken with a polarizing microscope. Hardly any birefringence in the zona glomerulosa, the zona fasciculata with clumps of coarsely birefringent particles.

negative for those tests for which the known steroid-producing tissues of the body are positive. The tests when positive show up in the adrenal cortex substances in addition to the steroid hormones. When these tests are negative, it seems likely that neither these substances nor the steroid hormones are present. Alternatives to this interpretation are first that the hypothetical steroid hormone of the X zone is produced and passed into the blood stream so quickly that it is not "caught" by fixation; or second that this hypothetical hormone is not elaborated in the same way as the steroids are thought to be in the permanent adrenal cortex. If either of these alternatives were true, then the fact that the X zone is negative to the tests used would not be conclusive. There is no evidence available which would decide the issue.

Short term injection of testosterone propionate had only a slight effect on the permanent cortex of the prepuberally castrated mouse. Based on the birefringence, which was lessoned as compared with the controls and for the most part coarse in nature, the indication is that the secretory activity of the cortex has diminished. For, in general, heightened activity is reflected in increased fine birefringence and diminished activity by increased coarse birefringence (Weaver and Nelson, 1943; Deane, Shaw and Greep, 1948). The changes in the results from the other tests, as compared with the normals, are not definite enough to be conclusive. Testosterone injections have, however a marked effect on the adrenal as judged by histochemical tests when these animals are hypophysectomized. In this case the changes consequent on hypophysectomy are hastened and the adrenals of the injected mice 9 days after operation have the appearance of those from mice about 15 days or so after operation (from another series Jones, 1948b and unpublished). The zona glomerulosa has a lessened content of substances positive for the histochemical tests as compared to the untreated hypophysectomized animals, although in the routine preparation the zone is hardly changed in the adrenals of mice from any of the experimental categories. Further experimentation is required before these results in the zona glomerulosa can be interpreted. In the zona fasciculata there is much coalescence of the big sudanophilic droplets, the Schiff reaction is weak, and the birefringent particles are all coarse and clumped together. It is clear that the atrophy of the zona fasciculata of the hypophysectomized animal has been increased by testosterone injection. It appears then that testosterone can have a direct atrophic effect on the permanent cortex in the absence of the pituitary.

The writer is unaware of any other observations on the results of androgen injections into hypophysectomized mice. In the rat Leonard (1944) reported that testosterone injected into hypophysectomized animals had an "adrenocorticotrophin-like" effect on the adrenals in that the adrenal atrophy normally consequent on the operation did

not occur and that the glands were heavier than those of the untreated hypophysectomized controls. The results of Leonard are surprising especially in view of the fact that exogenous androgens do not cause hypertrophy of the adrenals in either the rat or the mouse, indeed atrophy is the usual consequence (see Selye, 1939; Parkes, 1945). Also adrenal "castration hypertrophy" in the rat is removed by androgen injections (Hall and Korenchevsky, 1938). The earlier report on the results of testosterone injection into the hypophysectomized rat by Cutuly *et al.* (1938) is more in accord with the results with the mouse given here. They noted that the adrenals of hypophysectomized rats after androgen treatment had histologically the typical shrunken cortices characteristically following hypophysectomy although 5 out of 12 adrenals were heavier than those of hypophysectomized controls. It is interesting to note that in another species, the ground squirrel, Zalesky *et al.*, (1941), found that testosterone injections had no effect on the atrophy or weights of the adrenals of hypophysectomized animals. On the whole it seems clearly established that testosterone can have a direct effect on the "permanent" cortex of the adrenal (that is without pituitary mediation) and that this action in the mouse is in the direction of atrophy. The physiological significance, however, of the action of androgens on the adrenal of either the intact or the operated animal is unknown.


## SUMMARY

The adrenal X zone persists in the prepuberally castrated male mouse and the zone is destroyed by testosterone injections. Likewise, the X zone persists, although atrophic, in the adrenal of the hypophysectomized, prepuberally castrated mouse and it is swept away by testosterone injections. The action of the testosterone on the X zone of the normal adrenal seems, therefore, to be a direct one.

The X zone of the adrenal is negative to those histochemical tests for which the permanent adrenal cortex and other steroid-producing organs are characteristically positive. This is in agreement with the view that a steroid-type hormone is not secreted by the X zone.

Injections of testosterone into hypophysectomized animal hastens the changes, as judged histochemically, in the zona glomerulosa and zona fasciculata of the adrenal consequent upon the operation.


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# CONCENTRATION OF ADRENOCORTICOTROPHIC HORMONE IN RAT, PORCINE AND HUMAN PITUITARY TISSUE[1]

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THE DEVELOPMENT of an accurate and sensitive method for the assay of adrenocorticotrophic hormone (ACTH), namely, the adrenal ascorbic acid-depletion method of Sayers, Sayers and Woodbury (1948), has made possible the quantitative determintaion of the concentration of this trophin in pituitary tissue. The adenohypophyses of three species—rat, hog and human—have been analyzed in this regard.

## METHODS AND MATERIALS

*Technic for Quantitative Extraction of ACTH from Pituitary Tissue.* During the initial phases of this study, extracts of anterior pituitary were prepared by grinding or homogenizing the fresh tissue in cold 0.9 per cent NaCl solution made alkaline to 0.01 N with NaOH. The technic is similar to those described in the literature for the measurement of pituitary gonadotrophic hormone potency. When pituitaries from a large number of control groups of rats were extracted by this method and found to vary widely in ACTH content, the technic of grinding or homogenizing fresh tissue was abandoned as unsatisfactory. A simple method was subsequently devised for the quantitative extraction of ACTH from pituitaries. The glands were first frozen, then lyophilized, and the dry tissue ground to a homogeneous powder. Extracts of this material were very potent and uniform in ACTH activity. Examination of Table 2 shows that extracts prepared from tissue which was first homogenized and then lyophilized were at best only one-fifth as potent (antilog of M equal to 0.19) as extracts from pituitaries lyophilized before being ground (Table 2).

The routine procedure now employed in this laboratory for extracting ACTH from pituitaries is as follows: Approximately three-quarters of a milligram of dry, lyophilized pituitary are weighed on a micro-analytical balance to the nearest 0.005 mg. Larger samples may be employed according to the total weight of tissue available. The tissue is extracted at room temperature successively with three approximately equal portions of 0.9 per cent NACl solution made alkaline to 0.01 N with NaOH. The residue is centrifuged down after each extraction and the supernatant portions are combined. An

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example of a typical extraction follows: the weight of dry, donor pituitary tissue was 0.725 mg.; the total volume of extraction fluid was 0.5 ml. per 0.04 mg. of tissue (the largest dose employed per 100 gm. of recipient rat), and was equal in this instance to 9.1 ml.; the tissue was extracted successively with 3.1, 3.0 and 3.0 ml. portions of the extraction fluid; two dilutions were prepared from the combined extracts by the addition of 0.9 per cent NaCl solution so that the concentration of tissue per 0.5 ml. was 0.02 mg. and 0.01 mg. respectively. The recipient rats were injected with either 0.5 ml. of the original extract or 0.5 ml. of one of the dilutions thereof, per 100 gm. of body weight.

Extraction of ACTH from the pituitary appears to be complete. Whereas the thrice-extracted material prepared as above gave an appreciable adrenal cortical response in recipient rats at a dose level equivalent to 0.005 mg. per 100 gm., a fourth extraction of the residue failed to elicit a response at a dose level equivalent to 1.0 mg. per 100 gm.

*Source of Pituitaries.* Rat pituitaries were obtained as follows: Male Sprague-Dawley rats weighing 250 to 350 gm. were fasted 24 hours, anesthetized with sodium pentobarbital and exsanguinated by cutting the abdominal aorta; the pituitaries were removed within a minute after death. The anterior lobes, freed from posterior tissue, were frozen and lyophilized. The assays were conducted on pools of rat pituitaries.

Pituitaries were removed from the hogs not later than 10 minutes after death. Six porcine anterior lobes were pooled, frozen, lyophilized and ground to a homogeneous powder.

Anterior lobes of three human pituitaries were obtained through the courtesy of Dr. C. McNeil, Holy Cross Hospital, Salt Lake City. They were frozen within two hours after removal and lyophilization was started immediately after freezing. Human pituitary #1 was from a 76-year old male who died approximately 24 hours after a myocardial infarction. The pituitary was removed five hours after death. Human pituitary #2 was from a 58-year old male with a bronchogenic carcinoma; death was due to peritonitis from a perforated peptic ulcer. The pituitary was removed eight hours after death. A cyst of the pituitary was not included in the anterior lobe tissue taken for ACTH analysis. Human pituitary #3 was from a full-term, stillborn infant with erythroblastosis fetalis. The pituitary was removed approximately five hours after death.

*Reference Standards.* Two reference standards of ACTH activity were employed. Preparation 62AA was prepared from porcine pituitary tissue according to the method of Sayers, White and Long (1943). This protein behaved as a single component in the Tiselius apparatus and in the ultracentrifuge; it was free of lactogenic, growth, gonadotrophic and thyrotrophic activities. Preparation La-1-A, isolated from porcine glands, was supplied by Dr. John Mote of the Armour Laboratories. The data in Table 2 indicate that La-1-A is slightly more potent than 62AA. The slopes of the log dose-response curves of these two preparations are not significantly different.

*Assay Procedure.* Twenty-four hours after hypophysectomy the recipient assay rat was anesthetized with sodium pentobarbital and the left adrenal removed and prepared for the analysis of ascorbic acid. The extract to be assayed was injected via the tail vein, 0.5 ml. per 100 gm. body weight, and one hour later the right adrenal was removed and prepared for ascorbic acid

TABLE 1. ASSAY OF HUMAN PITUITARY #1 AGAINST LA-1-A BY THE DIFFERENCE METHOD

| Dose (micrograms per 100 gm. body weight) | | Concentration of adrenal ascorbic acid (mg. per 100 gm. of tissue) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Human Pituitary #1 | | | La-1-A | | |
| Human Pituitary #1 | La-1-A | Left Adrenal | Right Adrenal | Difference | Left Adrenal | Right Adrenal | Difference |
| 8 | 2 | 390 | 229 | 161 | 350 | 218 | 132 |
| 8 | 2 | 425 | 240 | 185 | 355 | 214 | 141 |
| – | 2 | — | — | — | 463 | 264 | 199 |
| 4 | 1 | 359 | 236 | 123 | 412 | 272 | 140 |
| 4 | 1 | 415 | 315 | 100 | 427 | 300 | 127 |
| 4 | 1 | 439 | 383 | 56 | 350 | 236 | 114 |
| 4 | – | 307 | 237 | 70 | — | — | — |
| 2 | 0.5 | 337 | 280 | 57 | 416 | 367 | 49 |
| 2 | 0.5 | 397 | 365 | 32 | 481 | 392 | 89 |
| 2 | 0.5 | 457 | 405 | 52 | 384 | 306 | 78 |

| | Human Pituitary #1 | La-1-A |
|---|---|---|
| $b=$ | 185.6 | 141.8 |
| $s=$ | 25.66 | 24.32 |
| $\lambda = s/b =$ | 0.138 | 0.172 |

$M = \bar{1}.2719$; ratio of potencies Human pituitary #1/La-1-A $=0.187$

$S_M = \pm 0.0242$; limits of error at $p=0.67$ are 95% to 106%

Range of potencies Human pituitary #1/La-1-A in 67 cases out of $100 = 0.177 - 0.198$

analysis. The ACTH potency of the extract is proportional to the decrease in adrenal ascorbic acid as measured by the difference between the concentration of ascorbic acid in the left (control) and right adrenal. The details of the method have been presented elsewhere (Sayers, Sayers and Woodbury, 1948).

The tissue was extracted on the morning of the assay and the extracts and dilutions thereof were kept at 0°C until assayed. Unknown and standard preparations were compared on the same day except in the cases of human pituitaries #2 and #3. Doses of unknowns were selected so that the responses obtained closely approximated those of the standard. It was therefore necessary to establish the approximate potency of the unknown extracts by *preliminary* assays before proceeding to the *final* quantitative analyses. A sample protocol of such a final assay, presented in Table 1, represents the assay of human pituitary #1 against a purified preparation of ACTH (La-1-A, Armour) used as a standard.

## RESULTS

In Table 2 are presented in condensed form the data from all final assays performed in this experiment; they were compiled in a manner similar to that for the data of Table 1. In no instance was there a significant difference between the slopes (b values) of the log dose-response curves of the substances compared. The probability, P, corresponding to Fisher's $t$, is either 0.2 or greater for each difference in the slopes.

The antilog of M in Table 2 gives the ratio of the potencies of unknown to standard. Of the three species examined, rat pituitary tissue (in terms of dry weight) has the lowest order of adrenocorticotrophic hormone potency; it is only 0.08 as potent as La-1-A. The hog and human pituitaries analyzed in this study are very potent; in terms of dry weight, they are one-fifth as potent as the purified standards.

Two pools of rat anterior lobes collected on different dates had the same potency of adrenocorticotrophic activity. The three human pituitaries, despite the marked differences in age and causes of death of the donors, were quite uniform in their content of ACTH.

## DISCUSSION

The lyophilization technic reported here permits a quantitative extraction of ACTH from pituitary tissue. This fact together with the fact that the assay method is highly specific (Sayers, Sayers and Woodbury, 1948) suggests that the values presented above for ACTH concentration in pituitary tissue are very close to the true values.

The grinding or homogenizing of fresh tissue, a procedure routinely employed in the preparation of extracts of pituitary for the assay of gonadotrophins, yields extracts which are at best only one-

TABLE 2. CONCENTRATION OF ACTH IN PITUITARY TISSUE

| Pituitaries compared | No. recipient rats | b | P | s | λ | M | Antilog of M | $S_M$ | Antilog $(M-S_M)$-Antilog $(M+S_M)$ |
|---|---|---|---|---|---|---|---|---|---|
| Rat[1] (homogenized) (16)[2] | 24 | 80 | 0.2 | 20.4 | 0.255 | $\bar{1}.289$ | 0.19 | ±0.113 | 0.15–0.25 |
| Rat[1] (lyophilized) (10) | 24 | 113 | | 25.4 | 0.224 | | | | |
| 62AA[1,3] | 12 | 169 | 0.3 | 20.0 | 0.119 | $\bar{1}.926$ | 0.84 | ±0.033 | 0.78–0.91 |
| La-1-A[1,3] | 14 | 196 | | 30.3 | 0.155 | | | | |
| Rat[1] (13) | 19 | 152 | 0.8 | 21.9 | 0.145 | $\bar{2}.911$ | 0.08 | ±0.043 | 0.07–0.09 |
| La-1-A[1] | 19 | 158 | | 17.8 | 0.112 | | | | |
| Rat[1] (19) | 12 | 149 | 0.3 | 26.3 | 0.177 | $\bar{1}.995$ | 0.99 | ±0.072 | 0.84–1.17 |
| Rat[1] (10) | 24 | 113 | | 25.4 | 0.224 | | | | |
| Porcine (6) | 10 | 112 | 0.3 | 11.4 | 0.102 | $\bar{1}.299$ | 0.20 | ±0.084 | 0.16–0.24 |
| La-1-A | 10 | 79 | | 22.3 | 0.282 | | | | |
| Human #1 | 9 | 186 | 0.4 | 25.7 | 0.138 | $\bar{1}.272$ | 0.19 | ±0.024 | 0.18–0.20 |
| La-1-A | 9 | 142 | | 24.3 | 0.172 | | | | |
| Human #2 | 10 | 124 | 0.7 | 29.0 | 0.234 | $\bar{1}.309$ | 0.20 | ±0.093 | 0.16–0.25 |
| La-1-A | 9 | 142 | | 24.3 | 0.172 | | | | |
| Human #3 | 6 | 156 | 0.7 | 19.5 | 0.125 | $\bar{1}.381$ | 0.24 | ±0.081 | 0.20–0.29 |
| La-1-A | 9 | 142 | | 24.3 | 0.172 | | | | |

b = slope of the log dose-response curve; P = probability factor measuring the significance of the difference between the slopes; s = standard deviation of all of the individual responses about this curve (a straight line fitted by the method of least squares); λ = s/b and is a measure of the accuracy of the assay method; M = logarithm of the ratio of the potencies; $S_M$ = standard error of M; antilog $(M-S_M)$-antilog $(M+S_M)$ is the range of estimate for one std. error.

[1] Several assays combined for statistical analysis.

[2] No. animals in donor groups in parentheses.

[3] La-1-A and 62AA are purified preparations of ACTH isolated from porcine pituitaries.

fifth as potent in adrenocorticotrophic activity as those obtained by the lyophilization technic. It would be of interest to compare the gonadotrophic potency of extracts of pituitary tissue prepared by "standard" methods with that of extracts prepared by the technic developed in this study.

Lyophilized pituitaries may be stored in a desiccator until such time as it is convenient to assay the ACTH activity. There has been no indication that a decrease in potency takes place over a period of at least one year when the tissue is stored in this manner.

The fact that there is no significant difference between the slopes of the log dose-response curves of purified porcine ACTH standards and of crude extracts of rat, porcine and human pituitaries suggests that the biological characteristics of adrenocorticotrophin in the several species and in crude and purified preparations are similar. Li *et al.* (1943) and Sayers *et al.* (1943) isolated from sheep and hog pituitaries, respectively, biologically pure and chemically homogeneous proteins having high adrenocorticotrophic activity. Both of these proteins had molecular weights of 20,000. However, reports have appeared in the literature to the effect that the hormone activity is dialyzable and ultrafiltrable (Tyslowitz, 1943; Crooke *et al.*, 1947). Li (1947), in a note lacking experimental details, claims that hydrolysis of ACTH yields active polypeptides with an average amino acid chain-length of seven. In this laboratory[2] it has been found that the adrenocorticotrophic activity of extracts of lyophilized pituitary tissue will not pass an ultrafiltration membrane whose pores are large enough to allow protamine to pass freely. Although it is fairly certain that the biological properties of isolated preparations of ACTH and of the hormone as it exists in pituitary tissue are identical, there is little certainty concerning the size of the protein (or of the moiety of protein) which carries the biological activity.

If it is assumed that the molecular weight of the hormone as it exists in the pituitary is 20,000, *i.e.*, the same as that of the standard 62AA, then one-fifth of the dry weight of porcine and human adenohypophyseal tissue consists of ACTH. The average dry weight of the anterior lobe of the adult hog is about 60 mg.; therefore this animal has a store of about 12 mg. of ACTH. The average dry weight of the anterior lobe of the adult human is about 100 mg.; therefore this species has a store of about 20 mg. of ACTH. Such large stores ensure a ready supply of adrenocorticotrophin in times of emergency. This is clearly illustrated by experimental data on the rate of discharge of ACTH from the pituitary during stress. Employing the adrenal ascorbic acid-depletion method for measuring rate of discharge of ACTH, Sayers and Sayers (1947) found that application of mild, short-acting forms of stress in rats resulted in a rate of discharge of

[2] Unpublished observations of John B. Richards, Thomas W. Burns and George Sayers.

ACTH equivalent to approximately one microgram of standard per hour. From the information in Table 2, the total ACTH in the pituitary gland of the rat can be calculated to be about 100 micrograms, a quantity far in excess of immediate needs of the animal when exposed to mild stress. However, following the application of severe stress, the content of ACTH in the rat pituitary may be reduced by one-half within a period of one hour (Sayers and Cheng, 1949). If this information is applicable to man, then a quantity of ACTH of the order of 10 mg. may be discharged from the adenohypophysis of the adult within a period of a few hours following a severe injury or acute metabolic disturbance.

## SUMMARY

A method has been devised for the quantitative extraction of adrenocorticotrophic hormone from pituitary tissue. The fresh tissue is frozen, lyophilized and ground to a homogeneous powder. The powder is extracted with 0.9 per cent NaCl solution made alkaline to 0.01 N with NaOH.

The concentration of ACTH in pituitary tissue has been expressed in terms of a purified standard isolated from hog pituitaries. Rat, porcine and human adenohypophyseal tissues, in the form of dry powders, are eight, 20, and 21 per cent as potent as the standard, respectively. The biological characteristics of the hormone, as it exists in the purified standard and in the glands of the three species examined, are similar.

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# THE PHYSIOLOGICAL ACTIVITIES OF THE STEREOISOMERS OF THYROXINE

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MOST workers have assumed that D-thyroxine will have a lesser physiological activity than the naturally occuring L isomer, although there is as yet no quantitative agreement on their relative activities. In 1928 Harington prepared the D & L isomers and their activities were measured by Gaddum (1929–30) and Salter, Lerman and Means (1935). Gaddum measured the oxygen consumption of rats and found the L isomer to be more effective than the D isomer which had one third to two thirds the activity, depending on the dose given. Using metamorphosis of the tadpole as the criterion of activity, essentially the same results were obtained. Salter, Lerman and Means (1935) assayed the same materials in human myxoedema patients and found them to be equally active. Foster, Palmer and Leland (1936), measuring the carbon dioxide production in guinea pigs, found the DL form to be only one half as active as the L form, whence they inferred D-thyroxine to be inactive. Reineke and Turner (1943) and Reineke, Turner, Kohler, Hoover and Beezley (1945) made similar measurements. Reineke and Turner (1945) determined the relative activities of L- & DL-thyroxine in inhibiting the increase in thyroid weight of rats and chicks receiving thiouracil, and in accelerating metamorphosis of tadpoles. In all these comparisons the DL form was found to be half as active as the L form, supporting the view that the D isomer was inactive. Griesbach and Purves (1945) showed that the smallest amount of thyroxine necessary to prevent thyroxine deficiency changes in the pituitary was sharply defined and could be used as a measure of thyroxine requirement. Using this method, Griesbach, Kennedy and Purves (1947) compared three different samples of L-thyroxine with DL-thyroxine. All three samples of L-thyroxine showed equal activity corresponding to about 1.5 times that of DL-thyroxine. This would make D-thyroxine about one third as active as L-thyroxine. Pitt Rivers and Lerman (1947) prepared D-thyroxine and compared this material with the L isomer in human myxoedema patients, finding it to have between one eighth and one tenth the activity of L thyroxine.

These results are summarized in Table 1.

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TABLE 1. ACTIVITIES OF D & L THYROXINE AS REPORTED BY DIFFERENT WORKERS

| Observer | Method | Forms compared | D:L Ratio found or calculated |
|---|---|---|---|
| Gaddum 1929/30 | $O_2$ consumption of rat<br>Metamorphosis of tadpole | D & L<br>D & L | 0.66–0.33:1<br>0.8 –0.5 :1 |
| Salter et al. 1935 | Human myxoedema | D & L | 1:1 |
| Foster et al. 1936 | $O_2$ consumption of guinea pig | DL & L | 0:1 calc. |
| Reineke et al. 1943 | $CO_2$ production in guinea pig | DL & L | 0:1 calc. |
| Reineke et al. 1945 | $CO_2$ production in guinea pig | DL & L | 0:1 calc. |
| Reineke et al. 1945 | Thyroid wt. of rat receiving thiouracil | DL & L | 0:1 calc. |
| | Thyroid wt. of chick receiving thiouracil | DL & L | 0:1 calc. |
| | Metamorphosis of tadpole | DL & L | 0:1 calc. |
| Griesbach et al. 1947 | Inhibition of pit. change in methylthiouracil rat | DL & L | 0.3 :1 calc. |
| Pitt Rivers et al. | Human myxoedema | D & L | 0.125–0.1 :1 |

We have now been able to make a direct comparison of D- & L-thyroxine, results of which are reported in this paper.

## MATERIAL AND METHODS

The thyroxine samples used were: L-thyroxine prepared by oxidation of L-diiodo-tyrosine according to the method of Harington and Pitt Rivers (1945), being part of the material used in a previous experiment (Griesbach, Kennedy and Purves 1947): DL-thyroxine prepared by alkaline hydrolysis of thyroid: D-thyroxine prepared by oxidation of D-diiodo-tyrosine. This material was part of that prepared by Pitt-Rivers and Lerman. Sufficient material for testing in three rats only was available. The L & DL samples were twice recrystallized as the mono-sodium salt. Solutions were prepared by dissolving the weighed material in an excess of 0.1 N sodium carbonate and diluting with normal saline so that the final solutions contained 7.5 $\mu$g. DL-, 5 $\mu$g. L- and 10 $\mu$g. D-thyroxine respectively per millilitre. The strength of the solutions was confirmed by iodine analysis.

Young male albino rats of 50–70 gms. weight were used. They were placed on a strict vegetarian diet and methylthiouracil was given as an 0.025% solution in the drinking water. They were weighed twice weekly and the thyroxine dosage was adjusted to the body weight. The thyroxine was given subcutaneously once daily for seventeen to twenty days. The pituitaries were then examined as described by Griesbach and Purves (1943).

## RESULTS

The smallest dose of DL-thyroxine which prevented the development of a pituitary basophilia was 2.3 $\mu$g. per 100 gms. per day. The

equivalent value of L-thyroxine was 1.5 μg. per 100 gms. per day. In the case of D-thyroxine 5 μg. per 100 gms. per day gave a pituitary basophil count of 9.6% and 4 μg. a count of 28.5%. The animal receiving 10 μg. per 100 gms. per day had a count of 4.1%. These pituitary basophils were small, filled with well stained granules, and there was a complete absence of activated basophils, a certain proportion of which is found in our control animals. The thyroid showed subnormal activity with flattened epithelium and dense colloid.

These results are summarized in Table 2.

TABLE 2. THE EFFECT OF GRADED DOSES OF THYROXINE STEREOISOMERS ON THE PITUITARY BASOPHIL PERCENTAGE OF METHYLTHIOURACIL TREATED RATS

| Form given | No. of animals | Dose μg/100gm/day | Mean pituitary basophil % ±S.E.M. |
|---|---|---|---|
| — | 20 | — | 30 |
| DL | 10<br>10<br>10 | 2.2<br>2.3<br>2.4 | 14.2±0.75<br>8.4±0.92<br>6.5±0.73 |
| L | 10<br>10<br>10 | 1.4<br>1.5<br>1.6 | 14.9±1.40<br>6.8±0.98<br>6.5±0.74 |
| D | 1<br>1<br>1 | 4.0<br>5.0<br>10.0 | 28.5<br>9.6<br>4.1 |
| Animals without thyroxine and methylthiouracil | | | 7–10 |

The ratio of activities D:L found by direct comparison is 0.30:1 and is also 0.30:1 as calculated from the L:DL ratio 2.3:1.5.

## DISCUSSION

In attempting to evaluate the different results obtained for the activities of thyroxine stereoisomers, it should be remembered that in most cases different methods, species and materials have been used. In one case, however, the same materials have been compared in the same species: Reineke and Turner's L-thyroxine in the rat (Reineke and Turner, 1943 and Griesbach, Kennedy and Purves, 1947). The conflicting results obtained here must be attributed to the use of different methods. The thyroid weight method seems to be much less sensitive than the pituitary response, for the basophils show considerable change when the thyroxine supplied is varied by 0.1 μg. in the vicinity of the end point. This should be contrasted with the dose interval of 0.5 μg. used by Reineke and Turner (1945). In the guinea pig, the apparent inactivity of the D isomer is based on observations that approximately the same increase in metabolism was produced by doses of DL-thyroxine twice those of the L isomer. The errors inherent in the use of this relatively insensitive method are such that a

small activity for the D isomer could not be detected with certainty. The results of Salter, Lerman and Means (1935) and Pitt Rivers and Lerman (1947) obtained in human myxoedema patients, while lacking in quantitative agreement, definitely establish the fact that D-thyroxine is physiologically active in man. Our work has shown that the same holds true in the rat.

## SUMMARY

The thyroxine requirement of the rat was found to be 2.3 $\mu$g. of DL-thyroxine per 100 gms. per day, 1.5 $\mu$g. of L-thyroxine and 5 $\mu$g. of D-thyroxine.

D-thyroxine has been shown to have biological activity in the rat equal to 0.3 times that of L-thyroxine.

This result has been obtained by direct comparison of the D & L isomers and also by calculation from the L:DL ratio.

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# INFLUENCE OF THYROXINE ON PROTEIN METABOLISM

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It is customary to regard the thyroid hormone as a stimulator of catabolism, in particular of protein catabolism. However, the impaired growth of human cretins, of thyroidectomized rats (Evans, Simpson, and Pencharz 1939) and goats (Reineke, and Turner 1941) as well as the resumption of growth resulting from thyroid medication indicate that the influence of thyroid hormone on metabolic processes is more complex. Growth is, of necessity, associated with a prevalence of anabolic processes, especially of proteins. The experiments reported in this paper were undertaken in order to determine, (a) whether thyroxine induces nitrogen retention (protein anabolism), and (b) if so, under what conditions anabolic or catabolic effects would predominate.

## MATERIAL AND METHODS

The rats used in most of the experiments were descendants of the Wistar Strain, bred by a local dealer; rats of the Sprague-Dawley strain were employed in some instances. Their weights varied between 200 and 250 grams. Thyroidectomy was performed by the usual anterior approach, and hypophysectomy by the parapharyngeal route. Experiments on operated animals were started three weeks following operation. However, in one experiment the animals were tube-fed constant amounts of diet prior to operation, on the day of operation, and in the post-operative period. In this experiment (Table 2) the post-operative period refers to days 14–21 following thyroidectomy.

All animals were tube-fed, using a number 10 french catheter. The "Mixed diet" described by Ingle *et al.* (Ingle, Ginther, and Nezamis 1943) was employed. Samples of the diet were frequently ashed and the N-content, determined by the Kjeldahl method, was found to be 16 mg. per ml. Each animal was gradually adapted to tube feeding, and the amount was fed which maintained weight during the control period. Thus the intake varied from one animal to another, but was kept rigidly constant for each rat during the control and experimental periods. Feedings were at 7:30 A.M. and 5 P.M., except for the hypophysectomized animals which were fed at 7 A.M., 3 P.M. and 10 P.M. All animals had free access to water.

Rats were kept in individual wire screen metabolism cages. Urine specimens were collected at 24 hour intervals and analyzed daily. Each collecting flask contained 1 gm. of citric acid. Feces were analyzed in some experiments; they were collected daily, kept in 30% sulfuric acid, and the N-content was determined on the pooled specimens of each control and experimental period.

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Nitrogen was determined in urine, feces, and diet by a micro-Kjeldahl procedure.

Rats were kept in a constant temperature room (approximately 72°F), and each was studied for a control period of 7 days, followed by an experimental period of equal length. All manipulations (weighing, injections) were performed daily at the same time.

Upon completion of the experiment all animals were killed and a complete autopsy performed. In the case of the thyroidectomized rats a careful search was made for thyroid remnants. The completeness of hypophysectomy was judged by inspection of the fossa and by gross and microscopic study of thyroids, testes, and adrenals. Any tissue suspect of being a thyroid or pituitary remnant was sectioned and examined microscopically. Experiments on animals incompletely thyroidectomized or hypophysectomized were discarded.

TABLE 1. EFFECT OF THYROXINE ON NITROGEN EXCRETION OF THYROIDECTOMIZED RATS

| Exp. No. | Daily dose of thyroxine μg. | Sex | Mean urinary N excretion mg. per 24 hrs.* | | Diff. mg. | "P" of Diff. |
|---|---|---|---|---|---|---|
| | | | Control period | Treatment period | | |
| 1 | 10 | M | 223 | 188 | −35 | 0.01 |
| 2 | 5 | M | 224 | 180 | −44 | 0.01 |
| 3 | 5 | M | 256 | 199 | −57 | 0.01 |
| 4 | 5 | M | 214 | 162 | −52 | 0.01 |
| 5 | 5 | M | 202 | 169 | −33 | 0.01 |
| 6 | 5 | M | 256 | 202 | −54 | 0.01 |
| 7 | 5 | M | 176 | 131 | −45 | 0.01 |
| 8 | 5 | M | 183 | 155 | −28 | 0.01 |
| 9 | 5 | M | 173 | 130 | −43 | 0.01 |
| 10 | 5 | M | 168 | 148 | −20 | 0.02 |
| 11 | 5 | M | 209 | 161 | −48 | 0.01 |
| 12 | 5 | M | 168 | 156 | −12 | 0.4 |
| 13 | 5 | F | 158 | 137 | −21 | 0.01 |
| 14 | 5 | F | 151 | 129 | −22 | 0.01 |
| 15 | 5 | F | 167 | 143 | −24 | 0.01 |
| 16 | 50 | M | 232 | 252 | +20 | 0.01 |
| 17 | 50 | M | 153 | 179 | +26 | 0.01 |
| 18 | 50 | M | 155 | 179 | +24 | 0.01 |

* Includes the first 24 hrs. of the latent period.

## RESULTS

In 14 of 15 thyroidectomized rats injected daily for 7 days with either 5 or 10 micrograms of thyroxine the urinary output of N decreased significantly. There was no significant change in weight during this period. When 50 or 100 micrograms of thyroxine were injected daily, N-excretion increased. Both the anabolic effect of the small doses and the catabolic effect of the larger doses became evident on the third day of treatment (Table 1).

When rats were fed a constant intake prior to and immediately following thyroidectomy, the "protein catabolic" effect of thyroidectomy became evident. The N-excretion, which was 78% of the N intake prior to operation rose to 88% after thyroidectomy and fell to 62% following replacement therapy with 5 micrograms of thyroxine (Table 2).

TABLE 2. INFLUENCE OF THYROIDECTOMY AND OF SUBSEQUENT THYROXINE TREATMENT ON N BALANCE

| Rat No. | N-intake mg. per 24 hrs. | Control period | | | Post-operative period[5] | | | Treated[6] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Urinary N excretion mg. per 24 hrs. | % intake ex-creted | Weight change gm. | Urinary N excretion mg. per 24 hrs. | % intake ex-creted | Weight change gm. | Urinary N excretion mg. per 24 hrs. | % intake ex-creted | Weight change gm. |
| 1[1] | 280 | 216± 8.7[4] | 77 | +10 | 215±53 | 77 | +6 | 205± 7 | 74 | +10 |
| 2[1] | 280 | 237± 20 | 84 | + 2 | 239±10 | 85 | +1 | 232±11 | 83 | + 3 |
| 3[2] | 280 | 235± 10.9 | 84 | + 5 | 262± 9.6 | 94 | −5 | 181±10.3 | 65 | +16 |
| 4[2] | 280 | 209± 16.3 | 75 | + 7 | 228±26 | 80 | +1 | 172±10.6 | 61 | +10 |
| 5[2] | 280 | 213± 19 | 77 | + 5 | 254±15 | 90 | −5 | 172± 6.5 | 61 | +10 |
| 6[3] | 280 | 213± 19 | 77 | + 5 | | | | | | |
| 7[3] | 280 | 214± 6.2 | 77 | + 6 | | | | | | |
| 8[3] | 280 | 209± 15.3 | 75 | + 8 | | | | | | |
| M | | | 78 | | | 88[7] | | | | |

[1] Sham operated.
[2] Thyroidectomized.
[3] Died at operation.
[4] Standard deviation.
[5] 14th–21st day post-operation.
[6] Thyroxine (5 $\mu$g.) daily.
[7] (P of difference less than 0.02.)

In contradistinction to the protein anabolic (N sparing) effect of small doses of thyroxine in the thyroidectomized animal, thyroxine in amounts of 5–10 micrograms had no influence upon N-balance in the intact rat. 50 or 100 micrograms of thyroxine, however, caused an increased output of urinary nitrogen, and weight loss.

Hypophysectomized rats injected with 5 micrograms of thyroxine for 7 days showed no change in N-excretion or weight. 100 micrograms of thyroxine injected daily for 7 days failed to induce an increase of N-excretion. In three of the nine rats injection of the large dose of thyroxine was followed by a slight decrease in N excretion (Table 3).

Fecal nitrogen and fluid intake and output were not changed by any of the experimental procedures used.

TABLE 3. EFFECT OF THYROXINE IN HYPOPHYSECTOMIZED RATS

| Exp. No. | Daily dose of thyroxine $\gamma$ | Mean urinary N excretion mg. per 24 hrs.* | | Diff. mg. | "P" of Diff. |
|---|---|---|---|---|---|
| | | Control period | Treatment period | | |
| 1 | None | 198 | 193 | − 5 | 0.6 |
| 2 | None | 199 | 203 | + 4 | 0.7 |
| 3 | None | 205 | 206 | + 1 | 0.9 |
| 4 | None | 216 | 208 | − 8 | 0.3 |
| 5 | None | 192 | 194 | + 2 | 0.8 |
| 6 | 5 | 197 | 204 | + 7 | 0.4 |
| 7 | 5 | 192 | 199 | + 7 | 0.3 |
| 8 | 5 | 185 | 197 | +12 | 0.1 |
| 9 | 5 | 230 | 220 | −10 | 0.3 |
| 10 | 5 | 207 | 216 | + 9 | 0.2 |
| 11 | 5 | 195 | 192 | − 3 | 0.7 |
| 12 | 100 | 175 | 181 | + 6 | 0.3 |
| 13 | 100 | 197 | 181 | −16 | 0.02 |
| 14 | 100 | 193 | 180 | −13 | 0.02 |
| 15 | 100 | 190 | 179 | −11 | 0.02 |
| 16 | 100 | 186 | 179 | − 7 | 0.2 |
| 17 | 100 | 192 | 186 | − 6 | 0.3 |
| 18 | 100 | 191 | 189 | − 2 | 0.7 |
| 19 | 100 | 186 | 186 | 0 | 1 |
| 20 | 100 | 184 | 190 | + 6 | 0.4 |

* Includes first 24 hours of the latent period.

## DISCUSSION

That thyroid hormone exerts a protein anabolic effect under certain circumstances was suggested by observation that young thyroidectomized rats (Evans, Simpson, and Pencharz 1939) and goats (Reineke and Turner 1941) and human cretins resume growth under treatment with thyroid hormone. A greater growth rate of intact mice given thyroxine has been demonstrated; in the latter experiments, however, the weight increase was almost completely accounted for by increased food intake (Koger, Hurst, and Turner 1942). Our experiments were carried out on force-fed rats, thus obviating the difficulties of interpretation resulting from change in food intake following thyroid administration in ad lib. fed animals. Studies of N-balance in human hypothyroids with food intake as nearly controlled as possible have suggested increased N-retention under thyroid medication (Janey 1918, Johnston and Maroney 1939, Wilkins 1945) but in long-term clinical experiments the maintenance of a constant intake is difficult to enforce.

Our experiments demonstrate that the thyroidectomized rat under conditions of constant intake (force-feeding) retains less N than does the normal rat, either because it cannot anabolize protein or because simultaneously occurring catabolic processes are increased. The finding of increased N excretion following thyroidectomy is in agreement with the results of Persike (1948). It should be stressed that the studies on thyroidectomized rats were performed three weeks after operation except as noted in Table 2. Consequently, the increased N excretion cannot be interpreted as resulting from an unspecific alarm reaction. Furthermore, N excretion following other relatively simple operations in the rat is either only slightly increased for a short time or shows no significant change.

Daily injection of 5–10 micrograms of thyroxine decreases the nitrogen excretion of the thyroidectomized rat, apparently even to a level lower than that of the intact animal. The secretion of thyroid hormone by the rats thyroid has been calculated to be approximately 5–10 microgram equivalents of thyroxine per day (Dempsey and Astwood 1943, Reineke, Mixner and Turner 1945). The fact that administration of such amounts of thyroxine exert a protein-anabolic effect suggests that the latter may be the physiological action of thyroid hormone.

On the other hand, large amounts of thyroxine, 50 or 100 micrograms per day, induce protein catabolism in intact and in thyroidectomized rats under the conditions of our experiments. In ad lib. fed rats, certain anabolic processes in the liver have been observed after a single injection of large amounts of thyroxine, 400 micrograms per 100 gm. body-weight (Sternheimer 1939.) The experimental conditions in Sternheimer's experiments and in ours differ too widely to permit close comparison but the possibility of anabolic processes in

the liver occurring simultaneously with an overall negative nitrogen balance is of some interest.

The protein anabolic effect of physiological doses of thyroxine was absent in the hypophysectomized rats. Since the hypophysectomized rat is physiologically almost "athyroid," one would expect an anabolic effect such as was observed in the thyroidectomized rats. This suggests that pituitary function may be a prerequisite for the protein anabolic effect of thyroid. This would be in agreement with the observation that the thyroidectomized-hypophysectomized rat does not resume growth under treatment with thyroid hormone (Evans, Simpson and Pencharz 1939). The nature of this interaction or synergism between thyroid hormone and the pituitary cannot be interpreted at this time. It is probably significant in this connection that in our experiments (on fed hypophysectomized animals) large doses of thyroxine failed to induce protein catabolism whereas protein catabolism has been induced by thyroxine in the fasting hypophysectomized dog (Soskin, Mirsky, Zimmerman and Crohn 1938) and in the fasting hypophysectomized phlorhizinized rat (Wells and Chapman 1941).

## SUMMARY

Experiments are reported on the influence of thyroxine on N excretion in force-fed rats kept on a constant food intake.

Thyroidectomy in the rat is followed by an increased output of urinary N. This is a late effect not to be interpreted as unspecific post-operative negative N balance (alarm reaction).

Small, physiological doses of thyroxine decrease N excretion in the thyroidectomized rat (protein anabolic effect). In the intact rat such doses of thyroxine have no influence on N balance.

Large amounts of thyroxine increase N excretion both in the intact and in the thyroidectomized rat (protein catabolic effect).

In the hypophysectomized rat small doses of thyroxine fail to exert a protein anabolic effect and large doses fail to exert a protein catabolic effect.

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# LACK OF EFFECT OF LACTOGENIC HORMONE UPON ORGAN WEIGHTS, NITROGEN AND PHOSPHORUS BALANCE, AND THE FAT AND PROTEIN CONTENT OF LIVER AND CARCASS IN MALE RATS GIVEN LACTOGENIC HORMONE

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PRIOR TO THE isolation of the lactogenic hormone of the anterior pituitary in homogeneous form there were many reports that non-purified preparations of this hormone exerted metabolic effects not directly associated with its role in lactation and luteotrophic action. The biologic properties of pure lactogenic hormone have not been fully studied. We have been especially interested in the report of Reiss (1947) that a preparation of lactogenic hormone caused a significant decrease in the fat of carcass and skin in the rat. In the present study the administration of lactogenic hormone for 10 days to the force-fed male rat failed to cause any significant change in the weights of liver, stomach, intestines, kidneys, testes, thymus and adrenals, in nitrogen and phosphorus balance, or in the fat and protein content of the liver and carcass.

## METHODS

Male rats of the Sprague-Dawley strain were maintained on a diet of Archer Dog Pellets until they reached a weight of approximately 300 gm. They were then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.) The technique of force-feeding and the diets used were modifications of those described by Reinecke, Ball and Saumels (1939). The diet was made according to Table 1. During the period of adaptation to force-feeding the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc. per day on the 5th day.

The animals were housed in an air-contidioned room in which the temperature was maintained at 74 to 78 degrees F and the humidity at 30 to 35 per cent of saturation. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol and 1 gram of

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TABLE 1. MEDIUM CARBOHYDRATE DIET

| Constituent | Grams |
|---|---|
| Cellu flour (Chicago Dietetic Supply) | 120 |
| Osborne & Mendel salt mixture | 40 |
| Diet yeast (Pabst) | 100 |
| Wheat germ oil | 10 |
| Cod liver oil | 10 |
| Vitamin K (2-methyl-1,4-naphthoquinone) | 100 mg. |
| Mazola oil | 200 |
| Casein (Labco) | 160 |
| Starch | 200 |
| Dextrin | 190 |
| Sucrose | 200 |
| Water to make total of | 2000 cc. |

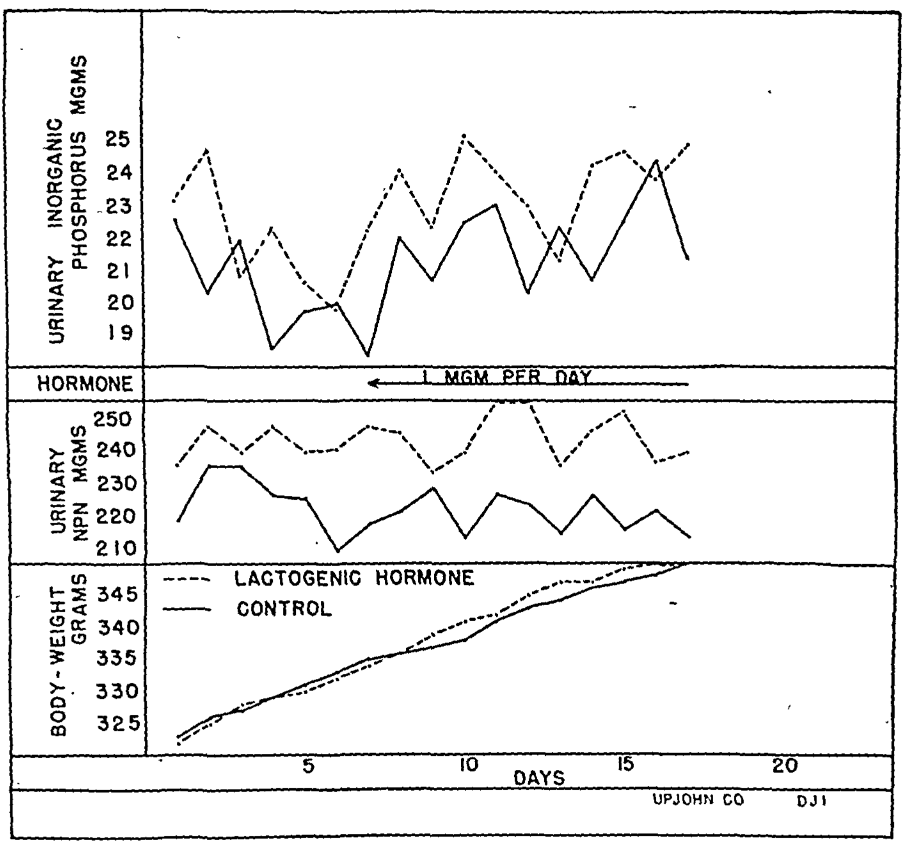


FIG. 1. Lack of effect of lactogenic hormone upon body-weight and urinary nitrogen and phosphorus in normal force-fed male rats. Averages.

citric acid to insure the acidity of the urines for nitrogen analysis. The following methods of analysis were used: Urinary inorganic phosphorus, Müller (1935); urinary non-protein nitrogen by the micro-Kjeldahl procedure; tissue fat and tissue protein, Li *et al.* (1948).

The lactogenic hormone was prepared by the procedure of Li, *et al.* (1942). Following control periods of 14 days, 8 experimental animals were each given 1 mgm. of lactogenic hormone by subcutaneous injection in 3 divided doses per day for 10 days. Eight control animals were given injections of physiological saline. At the end of the experiment the animals were anesthetized with ether and exsanguinated. The hair was carefully shaved from the body, all of the intra-thoracic and intra-abdominal organs were removed, and the carcass including skin was chilled but not frozen, ground and lyophilized at low temperature until dry. The liver was not ground but was subjected to lyophilization. The carcass and liver were then analyzed for fat and protein.

## RESULTS

The data on body-weight, urinary non-protein nitrogen and inorganic phosphorus are in Figure 1, the data on organ weights are in

TABLE 2. ORGAN WEIGHTS FROM LACTOGENIC HORMONE EXPERIMENT

| Organ | Lactogenic Hormone | | Controls | |
|---|---|---|---|---|
| | Average | Range | Average | Range |
| Liver | 9.95 gms. | 8.95–10.85 | 9.96 gms. | 8.40–11.00 |
| Stomach | 1.52 gms. | 1.45– 1.60 | 1.53 gms. | 1.40– 1.83 |
| Small Intestine | 5.95 gms. | 5.20– 6.45 | 5.89 gms. | 5.40– 6.34 |
| Cecum and Colon | 2.11 gms. | 1.85– 2.30 | 2.13 gms. | 2.00– 2.30 |
| Kidneys | 2.29 gms. | 2.10– 2.40 | 2.17 gms. | 1.85– 2.50 |
| Testes | 3.29 gms. | 2.98– 3.55 | 3.09 gms. | 2.70– 3.58 |
| Thymus | 242 mgms. | 105–334 | 269 mgms. | 184–330 |
| Adrenals | 44 mgms. | 38–48 | 46 mgms. | 40–52 |

TABLE 3. LIVER COMPOSITION OF RATS TREATED WITH LACTOGENIC HORMONE. AVERAGES AND RANGE

| | | Composition in Gms. per 100 Gms. Wet Liver | | |
|---|---|---|---|---|
| | Liver Wt. Gms. | Water | Fat | Protein |
| Lactogenic hormone | 9.95 (8.95–10.85) | 67.68 (66.9–68.6) | 5.39 (4.60–6.86) | 21.08 (19.0–22.2) |
| Control | 9.96 (8.40–11.00) | 67.55 (66.9–68.5) | 4.46 (2.86–5.92) | 22.06 (20.8–23.3) |

TABLE 4. CARCASS COMPOSITION OF RATS TREATED WITH LACTOGENIC HORMONE AVERAGES AND RANGE

| | | Composition in Gms. per 100 Gms. Wet Carcass | | |
|---|---|---|---|---|
| | Body Wt. Gms. | Water | Fat | Protein |
| Lactogenic hormone | 352 (339–363) | 68.67 (67.3–69.5) | 11.55 (8.6–13.6) | 15.36 (14.1–16.9) |
| Control | 350 (334–367) | 68.33 (65.9–70.7) | 12.19 (9.3–14.6) | 15.23 (14.1–16.2) |

Table 2, the data on liver composition are in Table 3 and the data on carcass composition are in Table 4. The administration of lactogenic hormone did not cause any significant change in any of the indices applied in this study. The animals treated with lactogenic hormone excreted more nitrogen than did their controls but his group difference was present during the pre-injection period so that it cannot be said that lactogenic hormone modified nitrogen balance.

## DISCUSSION

The differences between the negative results of this study and the positive effects of lactogenic hormone reported by Reiss (1947) must be due either to the presence of biologically active substances other than lactogenic hormone in the extracts used by Reiss or to differences in experimental procedures or the responsiveness of the test animals. Riddle and associates (1947) have summarized their extensive investigations of the biologic effects of "prolactin" in mammals and birds. They found that "prolactin" increases the weight of the liver and pancreas in doves and pigeons and increases the percentage of hepatic glycogen and fat in the pigeon. These actions were found to be absent or negligible in the rat and other mammals.

The data of Figure 1 shows a consistent average difference in the amounts of nitrogen excreted by the two groups during both the pre-injection and the experimental periods. This illustrates the advisability of using the data obtained during the control period to divide the animals into more precisely balanced groups than is likely to be attained by random selection.

## SUMMARY

Male rats of approximately 300 grams initial weight were force-fed a medium carbohydrate fluid diet. Following a control period of 14 days, 8 rats were given 1 mgm. of pure lactogenic hormone in 3 divided injections per day for 10 days. An equal number of control rats were given injections of physiological saline. Nitrogen and phosphorus were determined in urine during the experiment. At necropsy the liver, stomach, intestines, kidneys, testes, thymus and adrenals were weighed and the fat and protein content of the liver and carcass were determined. The results were negative.

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# STUDIES ON THE NATURE OF THE PROTEIN CATABOLIC RESPONSE TO ADRENAL CORTICAL EXTRACT[1,2]

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IT IS GENERALLY believed that in addition to their other actions, those adrenal cortical steroids which influence protein metabolism do so by increasing the rate of protein catabolism. The coupling of the increase in nitrogen excretion with an accumulation of liver glycogen following treatment with adrenal cortical hormones in the fasted animal has led to the concept that these steroids are concerned with the process of gluconeogenesis from protein (Long, Katzin, and Fry, 1940). These findings, amply substantiated by many different investigators, have directed attention toward various steps in the metabolic cycle between protein and glycogen as possible sites for the locus of action of the adrenal cortex in metabolism. The degradation of protein itself, the deamination of amino acids, the conversion of three carbon precursors to glucose and glycogen, the conversion of glucose to glycogen, and the inhibition of extra hepatic glucose utilization have all been considered in terms of the site of action of the cortical steroids in metabolism (Long 1942). The purpose of this study is to attempt to throw further light on the mechanism of intervention of the adrenal cortex in protein metabolism. In a previous study (Engel, Pentz, and Engel, 1948) a method was developed for measuring quantitatively small changes in protein metabolism over brief periods. The method depends on the relatively steady rate of urea synthesis that occurs during the first 18 to 24 hours following bilateral nephrectomy in the rat. Since urea is equally distributed throughout the body water, changes in the blood urea nitrogen of the nephrectomized rat after various experimental procedures quantitatively reflect increases or

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[2] Preliminary reports of portions of this work have appeared in abstract form, Engel, Pentz and Engel 1947 and Engel, Schiller, Pentz and Bondy 1948.

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decreases in protein catabolism, urea being the end product of this process.

By application of this technique to the study of cortical hormone action it has been possible to determine the time following injection at which the protein catabolic action of adrenal cortical extract becomes apparent and to measure quantitatively its magnitude. The effect of various experimental procedures on this action of cortical hormone will be considered.

## MATERIALS AND METHODS

Part of these experiments were carried out at Emory University and part at Duke University.

In the Emory series male rats of the Sprague-Dawley strain, weighing 180 to 240 grams were used. In the Duke series Vanderbilt strain male rats from 160 to 220 grams in weight were used. The former were fed Rockland rat chow, the latter Purina dog chow supplemented with liver pudding (pork liver 25, cornmeal 25, cracked wheat 25, powdered milk 3 and NaCl 1.4) and lettuce each twice a week. The Emory group were kept in an air-conditioned room from 24–27° C., the Duke group were not. The technique for studying nitrogen metabolism in the nephrectomized rat and the analytical methods have been described in previous reports (Engel and Engel, 1947, Engel, Pentz, and Engel, 1948). All results are expressed in milligrams of urea nitrogen synthesized per 100 grams body weight per hour, comparing a three hour control period with an equivalent experimental period. The animals were fasted for 16 hours following nephrectomy and prior to the experimental period. With the exception of the adrenal cortical extract which was given subcutaneously, all test substances were given intravenously via the external saphenous vein. The animals were kept under light nembutal anesthesia during the experimental period.

TABLE 1. COMPOSITION OF AMINO ACID MIXTURES

| VuJ-n | mg. per cc. | mg. N per cc. | "Non-Glycogenic VuJ" | mg. per cc. | mg. N per cc. |
|---|---|---|---|---|---|
| Glycine | 8.0 | 1.49 | Glycine | — | — |
| dl-threonine | 8.6 | 0.95 | l-threonine | 2.0 | 0.2 |
| dl-valine | 11.1 | 1.33 | l-valine | 6.5 | 0.87 |
| l-histidine-HCL | 3.2 | 0.64 | l-histidine-HCL-$H_2O$ | 3.4 | 0.68 |
| l-arginine-HCl | 6.4 | 1.70 | l-arginine-HCl | 2.5 | 0.81 |
| dl-phenylalanine | 5.5 | 0.47 | l-phenylalanine | 6.0 | 0.50 |
| dl-methionine | 4.9 | 0.45 | l-methionine | 3.8 | 0.35 |
| dl-isoleucine | 8.6 | 0.91 | l-isoleucine | 7.0 | 0.75 |
| dl-leucine | 12.3 | 1.31 | l-leucine | 15.0 | 1.60 |
| l-lysine-HCl | 9.9 | 1.34 | l-lysine-HCl-$H_2O$ | 61.2 | 8.53 |
| dl-tryptophane | 1.5 | 0.20 | l-tryptophane | 2.0 | 0.27 |
| Total | 80.0 | 10.79 | | 109.4 | 14.56 |
| At a dose of 0.87 ml. per 100 gms. (9.4 mg. N) 70% (5.5 mg.) of the utilizable N (7.8 mg. N) comes from glycogenic amino acids and 30% (2.3 mg.) from non-glycogenic amino acids. | | | At a dose of 0.81 ml. per 100 gms. (11.8 mg. N) 23.6% (2.8 mg.) of the N comes from glycogenic amino acids and 76.4% (9.0 mg.) from non-glycogenic amino acids. | | |

Liver glycogen levels were measured by a modification of the method of Good, Kramer and Somogyi (1933), with glucose determined by the photometric technique of Nelson (1944).

All test materials were injected as sterile solutions. The following substances were used:

1. VuJ-n (Merck and Company). An 8 per cent solution of synthetic amino acids consisting of the 10 essential amino acids plus glycine in the proportions listed in Table 1. This was used at a dose of 0.87 ml. or 9.4 mg. N per 100 gms. body weight. 70 per cent of the nitrogen in this mixture came from glycogenic amino acids. With the exception of glycine, histidine and arginine all the amino acids in this mixture were in the racemic form. On the basis of present information as to the utilization of d-amino acids in the rat, it was estimated that 83 per cent of the injected N could be used (Albanese, 1947).

2. "Non-glycogenic VuJ." A mixture prepared by ourselves from material supplied by Merck and Company and having the composition recorded in Table 1. But for glycine, it contained the same amino acids as the VuJ-n but all were of the natural form. The six mono-amino mono-carboxylic acids (l-isoleucine, l-leucine, l-methionine, l-phenylalanine, l-threonine and l-valine) were in form of a specially prepared casein hydrolysate and contained a small proportion of unidentified nitrogen. The solution was made up to a concentration of 10.9 per cent, adjusted to pH 7.05, autoclaved and injected at a dose of 0.81 ml. or 11.8 mg. N per 100 grams body weight. 23.6 per cent of the nitrogen in this mixture was derived from glycogenic amino acids. It should be noted that lysine was the chief non-glycogenic amino acid in the mixture.

3. Lysine-hydrochloride 6.85 per cent adjusted to pH 7.4 and autoclaved. Dose 1 ml. per 100 grams body weight (50 mg. free lysine or 9.6 mg. N).

4. 5 per cent glucose in normal saline. Dose 1 ml. or 50 mg. glucose per 100 gms. body weight.

5. VuJ-n in 5 per cent glucose in distilled water. Dose 0.87 ml. per 100 gms. body weight.

6. Human serum albumin, salt free, diluted to 6.25 per cent with normal saline. Dose 1 ml. or 10 mg. N per 100 gms. body weight.

7. 6.25 per cent human serum albumin in 5 per cent glucose in saline. Dose 1 ml. per 100 gms. body weight.

8. 15 per cent corn oil emulsion stabilized with 3 per cent soy bean phosphatides prepared by Dr. F. J. Stare. Diluted to 3 per cent with normal saline and administered at a dose of 1 ml. or 30 mg. of fat per 100 gms. body weight.

9. Upjohn's aqueous adrenal cortex extract.

## RESULTS

The results to be recorded represent data collected in two laboratories from different strains of rats kept under different dietary and environmental conditions. Although the basal hourly rates of urea formation calculated for a three hour period beginning 15–17 hours after nephrectomy were different in the two strains, the responses to the experimental procedures were qualitatively and quantitatively

similar in all cases compared. In 201 rats of the Emory series the basal rate was 3.0 ±0.08 mg. N per 100 gms. per hour (Engel, Pentz and Engel, 1948) while in 243 rats of the Duke series the rate was 2.0 ±0.05 mg. N per 100 gms. per hour. A third series of animals reported in the accompanying paper and studied in the Emory laboratory were of the Rockland strain and fed a low potassium diet. These animals had a basal rate of urea formation of about 1.8 mg. N per 100 gms. per hour (Bondy, Engel, and Farrar, 1949). The laboratory of origin of each experiment is recorded in each table.

*Effects of Adrenal Cortical Extract*

Adrenal cortical extract (ACE) was injected subcutaneously in two equal doses at the beginning and end of the first hour of the control period. Blood was collected at 0, 3, and 6 hours (16, 19, and 22 hours after nephrectomy) and the average hourly rates of urea for-

TABLE 2. INFLUENCE OF A.C.E. ON UREA N PRODUCTION IN THE NEPHRECTOMIZED RAT

| Procedure* | No. of rats | Rate of urea nitrogen formation in mg. N per 100 gm. body wt. per hr. | | | |
|---|---|---|---|---|---|
| | | 0–3 hrs. | 3–6 hrs. | Change | "p" |
| Saline (1.0 ml. per 100 gm.) | 10 | 1.7 ±0.15 | 1.9 ±0.17 | +0.2 ±0.20 | >0.5 |
| A.C.E. (0.5 ml. per 100 gm.) | 11 | 1.9 ±0.20 | 2.6 ±0.23 | +0.7 ±0.25 | <0.02 |
| A.C.E. (0.8 ml. per 100 gm.) | 17 | 1.8 ±0.22 | 2.6 ±0.28 | +0.8 ±0.29 | <0.01 |
| A.C.E. (1.0 ml. per 100 gm.) | 22 | 2.2 ±0.20 | 3.0 ±0.27 | +0.8 ±0.24 | <0.01 |
| A.C.E. (2.0 ml. per 100 gm.) | 12 | 1.8 ±0.20 | 2.8 ±0.27 | +1.0 ±0.32 | <0.01 |

* Duke Series—Basal Rate 2.0 ±0.05 mg. N per 100 gms. per hour.
The values in this and subsequent tables represent the means with their standard errors.

mation calculated for the 0–3 and the 3–6 hour periods. A control series in which 1 ml. of normal saline was injected is included. Table 2 records the results. Note that the saline was without effect on the rate of urea formation. Regardless of dose, the rate of urea formation during the first three hours following the injection of ACE was not significantly different from the basal control rate of 2.0 ±0.05 mg. N per 100 gms. per hour. This confirms previously reported data using a different strain of rats (Engel, Pentz and Engel, 1948).

During the second three hour period a highly significant increase in urea formation occurred with all doses of ACE used except the smallest. Within the dose range employed there was no clear-cut correlation between the dose and response once a significant increment

was achieved. From these studies it is apparent that the protein catabolic effect of ACE in the fasted nephrectomized rat begins about 3 hours after injection and amounts to about 1 mg. of N per 100 gms. body weight per hour for the subsequent 3 hours under the conditions of these experiments. This may be compared to the increase of 2.2 mg. of total urinary nitrogen per 100 gms. body weight per hour found by Long, Katzin, and Fry (1940) following 12 consecutive hourly injections of 1 ml. of ACE into normal fasted rats and 0.7 mg. of urinary nitrogen per 100 gms. per hour for 24 hours after 8 hourly injections of 1 ml. each of adrenal cortical extract found by Noble and Toby (1948). No attempts have been made to determine how long the effect lasts in our experiments. A similar stimulation of protein catabolism occurs after ACE in the adrenalectomized-nephrectomized rat (Bondy and Engel, 1947).

*Effects of Amino Acids on Protein Catabolic Action of ACE*

If the adrenal cortex were concerned primarily with the conversion of amino acids to glycogen, or if it had an obligatory effect on endogenous protein catabolism, one might anticipate that the administration of amino acids at a time when ACE was exhibiting its effect on nitrogen metabolism would result in an enhanced rate of urea formation. The data in Table 3 shows that this is not the case. 9.4 mg. of N in the form of a mixture of amino acids (Merck's VuJ-n) injected intravenously at the end of the third hour increased the rate of urea N formation 1.2 ±0.18 mg. per hour during the subsequent 3 hours. When combined with a small dose of ACE (0.4 ml. per 100 gms.) which itself caused no significant stimulation of protein catabolism, 1.3 ±0.20 mg. of urea N per hour appeared during the following 3 hours. Administration of VuJ-n to rats which had received doses of ACE causing increases in urea-N of the order of 1.2 ±0.28 and 0.9 ±

TABLE 3. INFLUENCE OF INTRAVENOUS AMINO ACIDS ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

| Procedure* | No. of Rats | Urea N mg. N per 100 gm. body wt. per hour | | | "p" |
|---|---|---|---|---|---|
| | | 0–3 hrs. | 3–6 hrs. | Change | |
| VuJ-n (9.4 mg. N per 100 gm.) | 24 | 3.1±0.23 | 4.3±0.23 | +1.2±0.18 | <0.01 |
| A.C.E. (0.4 ml. per 100 gm.) | 18 | 3.0±0.21 | 3.4±0.26 | +0.4±0.23 | 0.2 |
| A.C.E. (0.4 ml. per 100 gm. plus VuJ-n 9.5 mg. N/100 gm.) | 10 | 3.0±0.31 | 4.3±0.15 | +1.3±0.20 | <0.01 |
| A.C.E. (0.8 ml. per 100 gm.) | 13 | 2.9±0.22 | 4.1±0.34 | +1.2±0.28 | <0.01 |
| A.C.E. (0.8 ml. per 100 gm. VuJ-n 9.4 mg. N per 100 gm.) | 12 | 2.6±0.35 | 3.9±0.27 | +1.3±0.32 | <0.01 |
| A.C.E. (1.2 ml. per 100 gm.) | 12 | 2.8±0.21 | 3.7±0.26 | +0.9±0.23 | <0.01 |
| A.C.E. (1.2 ml. per 100 gm. VuJ-n 9.4 mg. N per 100 gm.) | 10 | 2.8±0.26 | 4.0±0.19 | +1.2±0.22 | <0.01 |
| VuJ-n (9.4 mg. N per 100 gm. i.v., 9.4 mg. N per 100 gm. i.p) | 9 | 3.4±0.20 | 6.1±0.31 | +2.7±0.26 | <0.01 |

* Emory Series—Basal Rate 3.0±0.08 mg. N per 100 gm. per hour.

0.23 mg. N per hour, yielded no greater amount of urea than if either ACE or the VuJ-n alone were injected. The increases were 1.3 ±0.32 and 1.2 ±0.22 mg. N per 100 gms. per hour, respectively. This represents conversion to urea of approximately 46 per cent of either the injected nitrogen or its equivalent from the tissues. This is not the limit of the liver's ability to deaminate amino acids under these circumstances as doubling the dose of amino acids approximately doubled the subsequent yield of urea. This is recorded in the last line of Table 3. The administration of 18.8 mg. of N per 100 gms. resulted in the formation of 2.7 ±0.27 mg. of extra urea N per hour for the subsequent 3 hours.

The failure to get an additive effect on urea formation when ACE and amino acids were administered concurrently argues against deamination of amino acids with subsequent urea formation as being a necessary result of cortical hormone action. By the same token, however, these results can be taken as evidence against a primary action on protein breakdown by ACE. One would anticipate that the amino acids resulting from this reaction would be added to the amino acid pool available for deamination by the liver, unless the high concentration of amino acids resulting from the injection suppresses endogenous protein catabolism. The following experiment suggests that the latter may be the case. In a previous study (Engel and Engel, 1946) it was shown that following the intravenous injection of amino acids into the nephrectomized rat most of the resulting urea was formed in the first hour, less in the second and very little in the third. Although the total amounts of urea formed in 3 hours after ACE, VuJ-n and ACE plus VuJ-n were the same it seemed possible that a difference in the rates of urea formation after each of these substances might be detected if measured each hour under these circumstances. The results are shown in Table 4.

The average hourly rates of urea formation for the control and experimental periods are recorded in the first part of the table. The second part indicates the excess urea above the basal rate formed during the 4th, 5th, and 6th hours respectively. Due to the greater amount of sampling and deeper anesthesia with resultant greater tendency to respiratory obstruction from mucus, a small but just significant increase in urea formation occurred during the second three hour period in the control animals. This did not occur in other experiments in which samples of blood were taken only at 3 hour intervals. The increase was of probable significance during the 3rd to 4th hour ($p < 0.05$) but not during the last two hours. The stimulation of urea formation by mild hemorrhage and anoxia has been noted before (Engel & Engel, 1946). It is reflected in subsequent experiments with ACE and VuJ-n by a somewhat greater urea formation than recorded in the previous experiment in which less bleeding was done. In the ACE treated rats a significant increase in urea formation occurred,

TABLE 4. UREA SYNTHESIS EACH HOUR AFTER A.C.E. AND AMINO ACID TREATMENT IN NEPHRECTOMIZED RATS

| Procedure* | No. of rats | Mg. Urea N per 100 gm. body wt. per hour | | | Urea N formed in excess over control period Mg. N per 100 gms. | | | | | Liver glycogen Mg. per 100 gms. liver |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | | Exp. | | | | | | |
| | | 0–3 hrs. | "p"† | 3–6 hrs. | (4th hr.) | "p"† | (5th hr.) | "p"† | (6th hr.) | |
| Control | 14 | 1.8 ±0.23 | <0.05 | 2.7 ±0.27 | 1.2 ±0.49 p <0.05‡ | 0.5 | 0.7 ±0.48 p =0.2‡ | 0.5 | 0.3 ±0.30 p =0.5‡ | 283 ±34 |
| A.C.E. (0.8 ml. per 100 gm.) | 16 | 1.8 ±0.22 | <0.01 | 3.4 ±0.31 | 2.2 ±0.54 p <0.01 | 0.4 | 1.6 ±0.50 p <0.01 | 0.5 | 1.3 ±0.38 p <0.01 | 231 ±37 |
| VuJ-n (10.4 mg. N per 100 gm.) | 17 | 1.9 ±0.18 | <0.01 | 4.1 ±0.25 | 3.9 ±0.62 p <0.01 | <0.05 | 2.2 ±0.52 p <0.01 | <0.02 | 0.5 ±0.41 p =0.3 | 483 ±88 |
| A.C.E. (0.8 ml. per 100 gm.) VuJ-n (10.4 mg. N per 100 gm.) | 15 | 2.4 ±0.20 | <0.01 | 3.9 ±0.19 | 3.2 ±0.48 p <0.01 | <0.01 | 1.0 ±0.32 p <0.01 | 0.5 | 0.5 ±0.43 p =0.2 | 546 ±77 |

* Duke Series—Basal Rate 2.0 ±0.05 mg. N per 100 gms. per hour.
† The "p" values refer to the probability of significance between each column.
‡ The "p" values below refer to the probability of significance compared to the 0–3 hour basal rate.

as before. The excess amounts of urea formed each hour represented a significant increase over the basal rate, but are not significantly different from each other. If these rates are corrected for the non-specific stimulation by subtracting the excess urea appearing each hour in the control series they become 1.0 mg., 0.9 mg., and 1.0 mg. N per 100 gm. during the 4th, 5th, and 6th hours respectively. Following amino acid injection significant increases in urea formation occurred during the 4th and 5th hours, but not the 6th and the rates each hour were each significantly less than the previous one. Corrected for the non-specific stimulation these rates become 2.7, 1.5, and 0.2 mg. N per 100 gms. When ACE and VuJ-n were given the results were identical with those following VuJ-n alone and in sharp contrast with the relatively steady rate of urea formation after ACE alone. The corrected values for the increases in urea formation were 2.0, 0.3, and 0.2 mg. N per 100 gms. per hour. While the responses to amino acids and to ACE plus amino acids were the same in that the excess in urea formation each hour was significantly less than the preceding hour, none of these were significantly different from the corresponding hour in the ACE series, in which the excess urea formed each hour was approximately the same. In view of the larger error inherent in taking hourly samples this is not surprising in series of this size. Limited significance can be attached, therefore, to the experiment as a whole. Considering each response by itself it can only be suggested that the response to ACE and amino acids is more as if amino acids alone were given, i.e. that the amino acids suppressed the action of the ACE. A significant increase in liver glycogen occurred in the rats treated with amino acids and ACE, but not with ACE alone. Since the apparent suppression of the protein catabolic effect of ACE occurred under circumstances in which there was an increase in liver glycogen the possibility that the availability of gluconeogenetic material may determine whether endogenous protein breakdown takes place after ACE treatment was considered. The experiments recorded in Table 5 were therefore carried out.

The greatest proportion of nitrogen (70 per cent) in VuJ-n comes from glycogenic amino acids. Using the same amino acids except for glycine, a mixture was prepared in which the proportions were so changed that 76.4 per cent of the N now came from non-glycogenic amino acids (Table 1). This mixture, when given in a dose of 11.8 mg. N per 100 gms. body weight was less well utilized or tolerated than the VuJ, the equivalent of 61 per cent of the injected nitrogen appearing as urea in 3 hours. When injected into the ACE-treated animals, the amount of urea formed was neither significantly greater than that after the amino acids alone, nor significantly less than the expected sum if the effects of the amino acids and ACE were additive (Table 5). There was a small but probably significant ($p < 0.05$) increase in liver glycogen in the rats treated with ACE and "Non-

glycogenic VuJ." Since this mixture still contained some glycogenic amino acids, the experiment was repeated using a single, non-glycogenic amino acid, lysine. This material was probably toxic since when injected at a dose of 9.6 mg. N. per 100 gm., 10.14 extra mg. of urea nitrogen appeared in 3 hours and the liver glycogen was significantly decreased. However, when given with ACE, slightly less urea was formed than with lysine alone, again indicating a suppression of the ACE action on protein catabolism. The liver glycogen after ACE and lysine was significantly greater than after lysine alone, but not greater than the control. It was significantly less than after ACE alone.

TABLE 5. INFLUENCE OF INTRAVENOUS AMINO ACIDS ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

| Procedure* | No. of rats | Urea N mg. N per 100 gm. body weight per hr. | | | "p" | Liver glycogen mg. per 100 gm liver |
|---|---|---|---|---|---|---|
| | | 0–3 hrs. | 3–6 hrs. | Change | | |
| Non-glycogenic VuJ (11.8 mg. N per 100 gm.) | 13 | 2.1±0.29 | 4.9±0.28 | +2.8±0.34 | <.01 | 293± 77 |
| A.C.E. (0.8 ml. per 100 gm.) | 17 | 1.8±0.22 | 2.6±0.28 | +0.8±0.29 | <.01 | 231± 37 |
| A.C.E. (0.8 ml. per 100 gm.) plus non-glycogenic VuJ | 13 | 1.9±0.16 | 5.1±0.39 | +3.2±0.33 | <.01 | 499± 60 |
| Control | 9 | | | | | 748±126 |
| A.C.E. (0.8 ml. per 100 gm.) | 9 | | | | | 1335±173 |
| Lysine (9.6 mg. N per 100 gm.) | 13 | 2.3±0.20 | 5.7±0.15 | +3.4±0.29 | <.01 | 487± 82 |
| A.C.E. (0.8 ml. per 100 gm.) plus lysine (9.6 mg. N per 100 gm.) | 14 | 2.5±0.27 | 5.3±0.28 | +2.8±0.26 | <.01 | 928± 91 |

* Duke series—Basal Rate 2.0±0.05 mg. N per 100 gm. per hour.

These results suggest that the inhibitory action of amino acids may not be related directly to their glyconeogenetic potency. However, elevated liver glycogen levels after lysine and ACE raise the possibility that in the presence of ACE, the non-glycogenic amino acids simply exchange with glycogenic amino acids in the tissue. The alleged unreactivity of lysine throws doubt on such a concept (Schoenheimer, 1942). Lysine on the other hand may simply serve as a non-specific stress in these experiments.

## *Effects of Glucose on the Protein Catabolic Action of ACE*

With the observation that amino acids apparently suppressed the action of ACE on endogenous protein metabolism, it became of interest to see whether other metabolites had a similar action. Table 6 records the effects of glucose. The experiment was performed twice, once in each laboratory, with identical results but only those from the Duke laboratory are recorded. Glucose was administered intravenously in a dose of 50 mg. per 100 grams, a dose approximately isocaloric with that of the amino acid used in the previous experiments. Glucose itself had no effect on urea formation but when given in conjunction with ACE completely abolished the increase in nitro-

gen customarily seen after treatment with this hormone. That the suppression of urea formation is at the level of protein breakdown rather than deamination of amino acids is suggested by the four last experiments in this series in which it is shown that glucose does not prevent the increase in urea that followed the injection of amino acids. This was true both after rapid and slow injection of the amino acids. The rate of delivery of amino acids to the liver therefore is probably not a significant factor in the response to glucose.

A similar effect of glucose on nitrogen metabolism after ACE was noted by Long, Katzin, and Fry (1940) in intact rats given 5 ml. of cortical extract intraperitoneally and 750 mg. of glucose by stomach

TABLE 6. INFLUENCE OF INTRAVENOUS GLUCOSE ON THE UREA PRODUCTION AFTER A.C.E. AND AMINO ACIDS IN THE NEPHRECTOMIZED RAT

| Procedure | No. of rats | Urea N Mg. N per 100 gm. body wt. per hr. | | | "p" |
|---|---|---|---|---|---|
| | | 0–3 hrs. | 3–6 hrs. | Change | |
| ƒGlucose (50 mg. per 100 gm. i.v.) | 6 | 1.7±0.28 | 1.8±0.17 | +0.1±0.38 | 0.5 |
| ƒA.C.E. (0.8 ml. per 100 gm.) | 17 | 1.8±0.22 | 2.6±0.28 | +0.8±0.29 | <0.01 |
| ƒA.C.E. (0.8 ml. per 100 gm.) plus glucose (50 mg. per 100 gm.) | 11 | 2.1±0.18 | 2.3±0.21 | +0.2±0.27 | 0.5 |
| *VuJ-n (9.4 mg. N per 100 gm.)† | 24 | 3.1±0.23 | 4.3±0.23 | +1.2±0.18 | <0.01 |
| *VuJ-n 9.4 mg. N per 100 gm.) +Glucose (50 mg. per 100 gm.)† | 14 | 3.7±0.32 | 5.4±0.25 | +1.7±0.41 | <0.01 |
| ƒVuJ-n (10 mg. N per 100 gm.)‡ | 8 | 2.3±0.30 | 4.2±0.21 | +1.9±0.43 | <0.01 |
| ƒVuJ-n (10 mg. N per 100 gm.) plus glucose (50 mg. per 100 gm.)‡ | 8 | 1.7±0.31 | 4.5±0.31 | +2.8±0.35 | <0.01 |

* Emory Series—Basal Rate—3.0±0.08 mg. N per 100 gm. per hour.
ƒ Duke Series—Basal Rate—2.0±0.05 mg. N per 100 gm. per hour.
† Test solution injected intravenously over 5 minutes.
‡ Test solution injected intraperitoneally in 6 divided doses over one hour.

tube. No change in nitrogen excretion occurred during the four hours of observation. In a later report (Long, 1942) an increase of only 5.4 mg. of urinary nitrogen per 100 grams in 12 hours was found after 11 hourly injections of 1 ml. of cortical extract were given with three feedings of glucose. This may be compared to the increase of 27 to 33 mg. of N previously reported in fasted normal, adrenalectomized and hypophysectomized rats given a similar dose of cortical extract (Long, Katzin, and Fry 1940).

*Effect of Fat on the Protein Catabolic Action of ACE*

The availability of a fat emulsion suitable for intravenous administration made it possible to examine the effect of fat on the protein catabolic effect of ACE. This material in the form of a 15 per cent emulsion of corn oil stabilized with 3 per cent soy bean phosphatides was kindly supplied us by Dr. F. J. Stare of the Department of Nutrition, Harvard Medical School. The material was diluted to 3 per cent fat with saline and injected at a dose of 30 mg. of fat per

100 grams body weight. The results are seen in Table 7. Neither the saline control nor the fat itself produced any change in nitrogen metabolism. ACE produced its usual effect and, in contrast to the results with glucose and amino acids, was not inhibited by the fat. There were small rises in liver glycogen in each of the experimental groups compared to the saline controls, but these were not statistically significant. Assuming that the injected fat was metabolized, and we have no proof of this from these experiments, it is apparent that merely supplying calories to the fasting animal is not sufficient to block the ACE effect on protein metabolism.†

TABLE 7. INFLUENCE OF INTRAVENOUS FAT ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

| Procedure* | No. of rats | Urea N mg. N per 100 gm. body wt. per hr. | | | "p" | Liver glycogen mg. per 100 gm. liver |
|---|---|---|---|---|---|---|
| | | 0–3 hrs. | 3–6 hrs. | Change | | |
| Saline (1.0 ml. per 100 gm.) | 10 | 1.7±0.15 | 1.9±0.17 | +0.2±0.20 | >0.5 | 458±133 |
| Fat (1.0 ml. 3.0% in saline) | 11 | 2.3±0.26 | 2.3±0.20 | 0.0±0.19 | >0.5 | 709±142 |
| A.C.E. (1.0 ml. per 100 gms.) | 10 | 2.2±0.24 | 3.0±0.30 | +0.8±0.23 | <0.01 | 672± 99 |
| A.C.E. (1.0 ml. per 100 gms.) plus Fat (1.0 ml. 3.0% in saline) | 8 | 1.5±0.31 | 2.5±0.18 | +1.0±0.19 | <0.01 | 683±143 |

* Duke Series—Basal Rate 2.0±0.05 mg. N per 100 gm. per hr.

### *The Effects of Protein on the Protein Catabolic Action of ACE*

The evidence so far has indicated that ACE does not act primarily in protein metabolism at the level of deamination of amino acids and that such actions as it may have on protein breakdown itself may be masked by the administration of glucose and amino acids. The next consideration was what would be the effect of exogenously administered protein on ACE action. For this purpose salt poor human albumin was used. It was injected at a dose approximately equivalent to that of the amino acids used i.e. 10 mg. N per 100 grams. Since repeated and identical positive results had been achieved with ACE alone the results are compared with an earlier representative series with ACE. In Table 8 it will be seen that the intravenous injection of human serum albumin did not cause any increase in urea formation in the control animals. However, when injected into the ACE treated animals an increase of 1.6 ±0.28 mg. N per 100 grams per hour occurred. This increase was questionably significantly greater than that after ACE alone ($p < 0.05$). This result should be compared to the lack of additive effect seen when amino acids are given to ACE treated rats. No increase in urea formation followed the injection of albumin in 5 per cent glucose into the control rats. Moreover, when ACE was given glucose abolished the extra urea formation previously seen following albumin injection (Table 8).

† Since this manuscript was submitted for publication it was discovered that the fat emulsion as originally made contained 4.5 per cent glucose. In the dilution used, i.e. 3% of the solution contained 9 mg. of glucose per ml., a negligible amount.

TABLE 8. INFLUENCE OF INTRAVENOUS ALBUMIN ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

| Procedure* | No. of rats | Urer N mg. N 100 gm. body weight per hour | | | "p" |
|---|---|---|---|---|---|
| | | 0–3 hrs. | 3–6 hrs. | Change | |
| A.C.E. (1.0 ml. per 100 gm.)† | 22 | 2.2±0.20 | 3.0±0.27 | +0.8 ±0.24 | <0.01 |
| Human serum albumin (6.25% in saline, 10 mg. N per 100 gm.) | 12 | 2.4±0.21 | 2.7±0.25 | +0.3 ±0.32 | >0.5 |
| Human serum albumin (6.25% in saline 10 mg. N per 100 gm.) A.C.E. (1.0 ml. per 100 gm.) | 13 | 1.9±0.13 | 3.5±0.25 | +1.6 ±0.28 | <0.01 |
| Human serum albumin (6.25% in 5% glucose and saline, 10 mg. N per 100 gm. | 7 | 1.8±0.20 | 1.9±0.31 | +0.1 ±0.41 | >0.5 |
| Human serum albumin (6.25% in 5% glucose and saline, 10 mg. N per 100 gm.) | 14 | 2.0±0.15 | 2.5±0.28 | +0.5 ±0.35 | >0.5 |

* Duke Series—Basal Rate 2.0±0.05 mg. N per 100 gm. per hr.
† See Table 2.

DISCUSSION

The results of these studies confirm the well documented fact that adrenal cortical extract increases the rate of protein catabolism in the fasted animal. An effect was detectable in the experiments as early as 3 hours after its administration. This is consistent with the results from other studies on cortical hormone activity such as dissolution of lymphocytes (Dougherty and White, 1944) but had not been demonstrated previously with respect to nitrogen metabolism. The magnitude of the increase in nitrogen metabolism recorded during the three hours of these experiments was somewhat less than obtained by Long, Katzin, and Fry (1940) who measured the 12 hour urinary nitrogen excretion in rats receiving 1 ml. of cortical extract each hour, but similar to that found by Noble and Toby (1948) using smaller amounts of ACE. In our experiments there was no significant increase in urea formation as the dose of cortical extract was raised, but the possible prolongation of the response beyond 3 hours by larger doses was not investigated.

In these studies the increase in urea formation in the fasted rat after ACE treatment was prevented by an injection of glucose or of amino acids, but not when either fat or whole protein were administered. This could be interpreted as indicating that glucose and amino acid inhibited protein catabolism. However, without the use of a tracer technique it is not possible to distinguish between an actual decrease in protein catabolism and an increase in the rate of metabolism of amino acids by a pathway other than deamination and urea formation. The failure of glucose to alter the rate of urea formation after amino acid injection suggests that deamination is not the locus at which glucose exerts its effect in decreasing urea formation after ACE, but rather that glucose spares protein.

The modification of the protein catabolic response to ACE by the various metabolites injected makes possible further consideration

of both the mode and site of action of the adrenal cortex in protein metabolism. Since the administration of adrenal cortical steroids causes an increase in liver glycogen in both the fasted and the fed animal the possibility may be suggested that the availability of glucose or of glycogen precursors may determine in some way whether an increase in endogenous protein catabolism will occur following ACE treatment. The absence of the increase in protein catabolism after ACE when either glucose or amino acids were given and the failure of fat, a poor glycogen precursor, to influence the catabolic response all would favor such a view. The finding that the non-glycogenic amino acids also apparently masked the protein catabolic action of ACE does not seem to support such an interpretation. However, these latter data must be interpreted with caution. While there was no significant increase in urea formation after ACE plus non-glycogenic VuJ-n, compared to after each alone, neither was the change significantly less than that expected were the two additive. Lysine alone, on the other hand, definitely did not yield an additive effect to that from ACE alone. The choice of lysine for these experiments, however, may not have been the best. It was determined by the availability and high solubility of lysine so that it could be given in a dose comparable to the VuJ-n. According to the data of Schoenheimer (1942) lysine does not exchange its nitrogen like most other amino acids. Furthermore lysine is said not to be deaminated in vitro by liver or kidney slices (Felix and Naka, 1940), although Borsook, Deasy, Hagen-Smit, Keighley and Lowy (1948) have recently reported on the formation of a-amino adipic acid from lysine in liver homogenates. Neuberger and Sauger (1944), furthermore, report that both nitrogen atoms of 1-lysine were converted to urea when fed to rats. In addition, administration of a single amino acid or an unbalanced mixture may throw a "strain" on the normal mechanisms of metabolism, yielding a different result from that when a balanced mixture is given. Both after "non-glycogenic VuJ" and lysine in these experiments a considerably greater amount of urea nitrogen was formed than after the more balanced VuJ-n mixture. This may be interpreted either as indicating a high conversion to urea of the injected amino acids or, what is more likely, a "toxic" effect of the amino acids with resultant stimulation of endogenous protein catabolism. Unless tagged amino acids are used it is impossible to distingguish between these two effects. A stimulation of endogenous protein catabolism by lysine with the release of potentially glycogenic amino acids from the tissues could be a factor in the apparent inhibition of the protein catabolic action of ACE. The increase in liver glycogen after ACE plus "non-glycogenic VuJ" and ACE plus lysine could be taken to support such a view-point. For this reason we cannot interpret these results to mean that amino acids need not be glycogenic to inhibit the protein catabolic action of ACE.

The intravenous administration of fat had no effect on nitrogen metabolism in these experiments and did not effect the response to ACE. Assuming that the fat was utilized during this time, this result would indicate that supplying calories in the form of fat, which is not readily converted to carbohydrate, does not interfere with the ACE action on protein metabolism. A small but not significant increase in liver glycogen occurred after the fat. The studies of McKibbin, Ferry, and Stare (1946) on this fat emulsion clearly indicate that this preparation is utilized by dogs and is capable of maintaining a positive nitrogen balance on a low diet protein. By incorporating radioactive trilaurin into the fat emulsion and measuring the output of radioactive $CO_2$ in exhaled air Geyer, Chipman and Stare (1948) have demonstrated that this emulsion is utilized immediately following intravenous injection.

Albumin was not converted to urea in 3 hours when injected into the nephrectomized rats, but when given to ACE treated rats an amount of urea was formed which was just significantly greater ($p < 0.05$) than that expected after ACE alone. Without the use of tracers it is of course impossible to determine whether the urea formed arose from the injected albumin or from tissue protein. This result therefore cannot be taken to either support or deny the hypothesis that the availability of glycogen precursors determines whether protein catabolism will take place after ACE treatment. The fact that the extra urea formation after albumin and ACE was abolished when glucose was given with the albumin indicates that this protein catabolism, regardless of origin, is subject to the same influences as is the endogenous protein catabolism which follows ACE treatment.

When taken in conjunction with the finding that the adrenalectomized rat shows the same increment in urea formation after amino acids as does the normal rat and does not convert injected plasma protein to urea (Bondy, Engel, and Farrar, 1949) the above data lead to the conclusion that the adrenal cortex must intervene in protein metabolism at the level of protein itself. This conclusion is supported by the fact that glucose does not depress urea formation from injected amino acids while it does suppress urea formation from ACE. Conversely, the failure to get an additive effect when amino acids and ACE are given and the normal response to amino acids in the adrenalectomized rat argue against ACE having a significant influence on deamination of amino acids. The findings of Wells and Kendall (1940) that the adrenalectomized phlorhizinized rat fed casein alone was able to metabolize this protein and excrete the same amount of nitrogen and glucose as the intact phlorhizinized rat and that of Segalloff (1946) that the adrenalectomized rat can be maintained in good condition on a high protein carbohydrate free diet likewise support the view that the adrenalectomized rat has no serious defect in amino acid metabolism. Protein taken in the gastrointestinal

tract is, of course, introduced into the metabolic pool as amino acids. The decrease in gluconeogenesis from amino acids in kidney tissue in vitro reported by Russell and Wilhelmi (1941) may represent a special case applicable chiefly to renal metabolism. Koepf *et al.* (1941) found no such defect in liver slices from adrenalectomized rats.

However, the mechanism by which ACE increases the catabolism of whole protein is by no means clear. The fact that this response is readily inhibited by glucose and amino acids indicates that protein breakdown is not an obligatory action of ACE and suggests that it may be secondary to events occurring elsewhere in the metabolic pathways of carbohydrate and fat. There is ample experimental evidence indicating that the adrenal cortex is concerned with the utilization of carbohydrate. The studies of Long (1940, 1942) confirmed by many other investigators have indicated that in the fed animal a decreased oxidation of carbohydrate must occur to explain the accumulation of glycogen and the increase in blood sugar that occurs after treatment with adrenal cortical steroids or adrenocorticotrophic hormone. Nitrogen excretion in the fed animal or patient given adrenal cortical steroids or adrenocorticotrophic hormone varies from no change to moderate increases as the dose of hormone is increased, but in no case has been sufficient to account for the accumulation of carbohydrate that occurred (Ingle, Li, and Evans, 1946, Forsham, Thorn, Prunty, and Hills, 1948, Conn. Louis, and Wheeler, 1948). Pancreatic diabetes is alleviated by adrenalectomy (Long and Lukens, 1936). When account is taken of the decrease in food intake which follows adrenalectomy it is apparent that the improvement in the diabetes must be attributed more to increased utilization of carbohydrate in the absence of the adrenal cortex than to decreased gluconeogenesis from protein (Ingle and Prestrud, 1948).

On the basis of the data presently available, one might be tempted to speculate that the primary site of action of the adrenal cortex in metabolism is on carbohydrate metabolism, decreasing the oxidation of glucose and directing glucose disposal to glycogen storage and possibly conversion to fat. In the fasted animal this would make necessary the breakdown of protein to supply the precursors for glycogen and fat, while at the same time fat would be mobilized and oxidized to supply the calories not readily available from carbohydrate. In the fed animal the response to cortical steroids would depend on the caloric intake and the dose of hormone. Under optimal circumstances the materials supplied in the diet would obviate the need for extra endogenous protein catabolism to meet the change in metabolism. With larger doses of hormone or an indaequate dietary intake the tissue proteins might still be called on. Our observation that glucose and amino acids prevent the breakdown of protein is consistent with such a view since they would represent the substrates necessary to carry the process forward without calling on tissue protein. *For*

reasons already noted the results with the non-glycogenic amino acids which seem contradictory cannot be considered as conclusive. One might anticipate that other 3-carbon intermediaries such as lactate and pyruvate might be equally effective, but this experiment has not been done. The administration of fat might influence the pathway from carbohydrate to fat by the mass action law but would have little effect on glycogen synthesis. For this latter reaction another precursor, tissue proteins, would still be required. Whole protein intravenously might be anticipated to be called on in the same way as tissue protein and its utilization rendered largely unnecessary by glucose.

Although our data are readily explained by the above hypothesis there are reasons for not rejecting the possibility that the adrenal cortex may in addition play a role directly in the metabolism of protein. The adrenalectomized animal can still draw readily from protein in the diet and avoid hypoglycemia, but cannot do so readily by increasing tissue protein catabolism and gluconeogenesis, immediately after adrenalectomy, although later it can (Ingle and Oberle, 1946 Noble and Toby, 1948). The adrenalectomized-nephrectomized rat forms urea at a relatively constant rate from the time of operation to death, whereas the control nephrectomized rat catabolizes protein and makes urea at an ever increasing rate until death (Bondy and Engel, 1947). The adrenalectomized animal cannot mobilize protein in response to a stress as does the normal animal, and does not utilize injected plasma in the same way as the normal rat (Bondy, Engel, and Farrar, 1949). The adrenalectomized mouse does not draw on its lymphoid tissue nitrogen during a prolonged fast (White and Dougherty, 1948). It would appear possible that the adrenal cortex may be concerned both with the mobilization and translocation of protein and the catabolism of this protein. The magnitude of the latter reaction may depend on the relative availability of extra carbohydrate, amino acids or other precursors of carbohydrate. During fasting or when fat alone is available protein catabolism will of necessity occur, whereas if there is a plethora of glucose or amino acids protein catabolism will be inhibited. Further investigation is still necessary, however, to place clearly the protein catabolic action of the adrenal cortex in relation to the other changes in metabolism.

## SUMMARY

An increase in urea formation begins 3 hours after the subcutaneous administration of adrenal cortical extract to fasted nephrectomized rats.

This increase is prevented by glucose injected intravenously three hours after the cortical extract.

Injection of a mixture of the ten essential amino acids plus glycine resulted in no greater amount of urea formation in the nephrectomized rat when given in conjunction with adrenal cortical extract than

when either the amino acids or the adrenal cortical extract alone were injected. Data are presented to suggest that the amino acids actually inhibited the breakdown of endogenous protein which usually follows the injection of adrenal cortical extract into the fasted animal.

Similar results were obtained if the amino acid mixture was modified to contain predominantly non-glycogenic amino acids or if lysine alone was injected.

Intravenous fat administration did not inhibit the increase in urea formation after adrenal cortical extract.

Human serum albumin injected at the same dose level in terms of nitrogen as the amino acids did not stimulate urea formation in the nephrectomized rat. In conjunction with adrenal cortical extract, however, a slightly greater amount of urea was formed than after cortical extract alone. This effect was abolished if glucose was given with the albumin.

These data are interpreted to indicate that (a) the action of the adrenal cortex in nitrogen metabolism is on whole protein rather than amino acids and (b) that the increase in protein catabolism after adrenal cortex extract is readily modified by changing the internal metabolic environment. It is suggested that the amount of glucose or of glycogen precursors available may be a determining factor in whether protein catabolism will be stimulated by the adrenal cortex. The interpretation of the role of the adrenal cortex in protein metabolism as entirely secondary to changes in carbohydrate and/or fat metabolism is considered but held not to be adequate in light of presently available data. The suggestion is made that the adrenal cortex may be necessary to make protein available for catabolism but that the latter reaction occurs only if no other substances capable of readily entering the metabolic pathways of carbohydrate are available.

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# THE METABOLISM OF AMINO ACIDS AND PROTEIN IN THE ADRENALECTOMIZED-NEPHRECTOMIZED RAT[1]

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IN THE PRECEDING paper data were presented on the nature of the protein catabolic effect of adrenal cortical extract in nephrectomized rats (Engel, Schiller and Pentz, 1949). It was shown that the intravenous injection of glucose or amino acids 2 to 3 hours after adrenal cortical extract inhibited or masked the usual stimulation of nitrogen metabolism that occurred after administration of this hormone. It was further demonstrated that cortical hormone did not stimulate the conversion of injected amino acids to urea whereas it did stimulate urea formation after the injection of albumin. The latter effect was inhibited if glucose was given with the protein. It was concluded that in its action on protein metabolism and gluconeogenesis the adrenal cortex did not detectably influence the rate of deamination of amino acids but did intervene at the level of protein degradation itself.

The present study on the metabolism of amino acids and proteins in the adrenalectomized-nephrectomized rat was designed to throw further light on this problem. By the use of the adrenalectomized rat it is possible not only to examine the effects of the absence of the adrenal itself. but also to evaluate the possible role of non-specific stimulation of protein metabolism as a result of the experimental procedures in the animals with intact adrenals.

## MATERIALS AND METHODS

Male rats from Rockland farms, weighing between 180 and 240 grams, were fed a low potassium diet (Bondy and Engel, 1947) for three days before the experiment. Bilateral nephrectomy or adrenalectomy and nephrectomy were performed, and the animals injected with 2.5 mg. of desoxycorticosterone acetate in oil subcutaneously at the time of operation and at 18 or 40 hours postoperatively. All food was withheld, but water and saline were

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offered freely. The rats were bled from the tail at the time of operation, and at 18, 21, and 24 hours, or at 40, 43, and 46 hours after operation. The blood samples were analyzed for urea by the xanthydrol precipitation method of Engel and Engel (1947). At 21 or 43 hours, the test substance was administered by intravenous injection into the internal saphenous vein, which was exposed by a skin incision. The injection took five minutes for completion. Light nembutal anesthesia was used for operation and during the period of bleedings. The rate of urea formation was calculated from the increase of the blood urea level, assuming that urea was equally distributed throughout the total body water (63% of the body weight). Rates of urea formation were expressed as milligrams nitrogen per 100 grams body weight per hour. Most of the test substances injected were the same as in the previous report and administered in the same doses (Engel, Schiller and Pentz, 1949). In addition rat plasma was prepared in the following manner: At the end of the experiments, rats were killed by decapitation and their blood collected in heparin. The plasma was separated and frozen. When an adequate amount had accumulated, it was thawed, filtered through a Seitz filter into a sterile cellophane bag, and dialyzed for 12–18 hours against cold running tap water, then against 0.5 per cent sodium chloride solution for one hour. The bag was hung in the blast of an electric fan in a cold room until the contents had concentrated to less than their original volume. The preparation was analyzed for nonprotein nitrogen and total protein, and diluted with distilled water until the concentration of protein approximated 13 mg. of protein nitrogen per ml. The material was then refiltered, and stored under aseptic conditions. One hundred units of penicillin and 1:1000 merthiolate were added. The plasma thus obtained was sterile on culture and had no pyrogens when tested in rabbits. The quantity administered was adjusted to supply 10.4 mg. of protein nitrogen per 100 grams of rat. In some experiments glucose was added to the plasma to a concentration of 6 per cent.

## RESULTS

In a previous report from this laboratory (Bondy and Engel, 1947) it was shown that in order to achieve a reasonable period of survival after combined adrenalectomy and nephrectomy, it was necessary to deplete the animals of potassium for several days preoperatively. Under these circumstances the adrenalectomized-nephrectomized rat actually survived longer than the nephrectomized rat similarly pretreated with a low potassium diet and with injections of desoxycorticosterone acetate postoperatively. The nitrogen metabolism of the adrenalectomized animals was characterized by a relatively constant rate of urea accumulation from the time of operation until death whereas there was a rapidly accelerating rate in rats with intact adrenals. Since it appeared possible that the pattern of protein metabolism might be somewhat different shortly postoperatively in the rats with intact adrenals, compared to a later interval when urea formation had sharply increased, the present experiments were carried out at two intervals, namely 18 to 24 hours and 40 to 46 hours postoperatively. The average hourly rates of urea formation from

operation to the 18th and 40th hours, and from the 18th to 21st, and 40th to 43rd hours were compared in different series of nephrectomized rats with and without adrenals. During the 21st to 24th and the 43rd to 46th hours the effects of the various test materials were examined. The results of these experiments are shown in Tables 1 and 2.

TABLE 1. EFFECTS OF AMINO ACIDS, PLASMA PROTEIN, A.C.E. AND GLUCOSE ON UREA FORMATION IN NEPHRECTOMIZED AND ADRENALECTOMIZED-NEPHRECTOMIZED RATS ON LOW POTASSIUM DIET 21 HOURS POSTOPERATIVELY

| Procedure | No. of rats | Rate of urea N formation, mg. N per 100 gm. body weight per hr. | | | | |
|---|---|---|---|---|---|---|
| Hours postoperatively | | 0–18 hrs. | "p" | 18–21 hrs. | "p" | 21–24 hrs. |
| Nephrectomized VuJ-n* (9.4 mg. N per 100 gm.) | 16 | 2.7±0.11 | <0.01 | 1.8±0.18 | <0.01 | 3.0±0.32 |
| "p" | | <0.02 | | N.S. | | N.S. |
| Adrenalectomized-nephrectomized VuJ-n* (9.4 mg. N per 100 gm.) | 17 | 2.3±0.11 | <0.01 | 1.3±0.18 | <0.01 | 2.9±0.35 |
| Nephrectomized plasma* (10.4 mg. N per 100 gm.) | 6 | 2.4±0.13 | <0.01 | 1.6±0.24 | <0.02 | 2.6±0.24 |
| "p" | | N.S. | | <0.01 | | <0.01 |
| Adrenalectomized-nephrectomized plasma* (10.4 mg. N per 100 gm.) | 7 | 2.5±0.16 | <0.01 | 0.7±0.12 | N.S. | 0.8±0.30 |
| "p" | | N.S. | | N.S. | | <0.02 |
| Adrenalectomized-nephrectomized ACE† (0.8 ml. per 100 gm.) plasma* (10.4 mg. N per 100 gm.) | 8 | 2.3±0.15 | <0.01 | 1.0±0.18 | <0.01 | 2.1±0.35 |
| Adrenalectomized-nephrectomized ACE† (0.8 ml. per 100 gm.) | 3‡ | 2.6 | | 2.2 | | 2.5 |
| Nephrectomized normal potassium diet. Plasma* (10.4 mg. N per 100 gm.) | 7 | | | 2.3±0.31 | N.S. | 2.6±0.32 |
| Nephrectomized glucose* (50 mg. per 100 gms.) | 12 | 2.9±0.14 | <0.01 | 1.8±0.13 | N.S. | 1.7±0.32 |
| "p" | | N.S. | | N.S. | | N.S. |
| Adrenalectomized-nephrectomized glucose* (50 mg. per 100 gms.) | 10 | 2.7±0.10 | N.S. | 1.7±0.45 | N.S. | 1.5±0.36 |

* Administered intravenously at the 21st hour.
† Administered subcutaneously at the 18th and 19th hours.
‡ Number of animals too small for statistical analysis.

The rate of urea production immediately after operation was higher than the rate from 18–21 hours. The change was significant in all groups except in the adrenalectomized rats injected with glucose at 21 hours. In this series, the decrease of the mean was as large as in some of the other series, but the large standard error of the 18–21 hour period brought the value of "p" higher than 0.05. The higher postoperative rate of urea production may be related to the trauma of operation. Nowever, it is of interest that this higher rate was not observed in rats having a normal potassium intake in the diet (Engel, Pentz, and Engel, 1948).

After the injection of the amino acid mixture, VuJ-n, there was a significant increase in the rate of urea production both in animals with and without adrenals, in the 18 hour series. The increment was slightly larger in the adrenalectomized group, possibly because of the

somewhat lower initial (18–21 hour) rate of urea production in this series. No significant difference was found between the adrenalectomized animals and those with intact adrenals in their ability to increase their rate of urea production after the administration of amino acids. The final rates of urea formation in rats with and without adrenals were identical. It may be noted that the increment produced by VuJ-n in this experiment is the same as that previously reported in a different series of animals but on a chow diet and with intact adrenals, similarly injected in this laboratory (Engel, Pentz and Engel, 1948). These results confirm Evans' finding (1941) that the adrenalectomized-nephrectomized rat shows no impairment of ability to deaminate injected alanine.

The above results are in contrast to those obtained when the nitrogen was injected in the form of whole protein (rat plasma). After the administration of rat plasma there was no change in the rate of formation of urea in the adrenalectomized animals. Animals with intact adrenals, however, increased their rate of urea production significantly. The rate of urea formation was increased from 1.6 ±0.24 in the control period to 2.6 ±0.24 mg. per 100 gm. body weight per hour during the three hours following the injection of plasma. There was a significant difference in the rate of production of urea after the injection of plasma in adrenalectomized as compared to non-adrenalectomized animals. The increase in urea formation after plasma injection into nephrectomized rats with intact adrenals differs from the results described in the previous report (Engel, Schiller and Pentz, 1949). No change in urea formation was noted after human serum albumin treatment in rats similarly nephrectomized but previously maintained on a diet adequate in potassium unless adrenal cortical extract was given. The studies in this laboratory were suspended before it was possible to test the effect of albumin in the above strain of rat on a low potassium diet. However, one of us (F.L.E.) has studied the effect of intravenous plasma on the rate of urea formation in nephrectomized rats on a diet of normal potassium content in the Duke laboratory. The rate of urea formation was 2.3 ±0.31 mg. per 100 gms. body weight prior to plasma injection and 2.6 ±0.32 mg. per 100 gms. following treatment, an insignificant change (Table 1). This corresponds to the results with albumin in the same strain of rats under the same experimental conditions. It is conceivable, therefore, that the potassium deficiency is responsible in some way for the increase in urea formation after plasma.

These results imply that the adrenal cortex is necessary for the increase in the rate of urea production in fasting potassium depleted rats after the injection of rat plasma. In order to confirm this hypothesis, adrenalectomized-nephrectomized rats were injected with adrenal cortical extract 2 and 3 hours before the injection of plasma. In Table 1 it may be seen that, under these circumstances, there was

a significant increase in the rate of production of urea after the injection of plasma, the rate increasing from 1.0 ±0.18 to 2.1 ±0.35 mg. per 100 gm. body weight per hour. No significant differences were found between the rates of urea formation in the rat with intact adrenals and the adrenalectomized rat given adrenal cortical extract. The difference in the rate of urea formation after the administration of adrenal cortical extract and plasma to the adrenalectomized rat was significant as compared to the response to plasma alone, without the hormone.

It was desirable to separate the effect of adrenal cortical extract on the rate of urea synthesis in the adrenalectomized rat from the effect of the injection of plasma. A series of adrenalectomized rats was therefore given adrenal cortical extract without plasma; however limitations of time did not permit finishing this series. The incomplete results are shown in Table 1. It appears that the effect of the injection of adrenal cortical extract into an adrenalectomized rat is intermediate between the result when plasma alone is administered, and when both plasma and adrenal cortical extract were given.

A series of adrenalectomized-nephrectomized and nephrectomized rats were injected at 21 hours with glucose . This experiment did not produce any significant alteration in the rate of urea production in either group. It may therefore be accepted as a control observation, indicating that the handling of the rats and the injection of the volumes of fluid involved in the present experiments do not themselves influence the rate of urea synthesis. It may be noted that the lack of

TABLE 2. EFFECTS OF AMINO ACIDS, PLASMA AND GLUCOSE ON UREA FORMATION IN NEPHRECTOMIZED AND ADRENALECTOMIZED-NEPHRECTOMIZED RATS ON A LOW POTASSIUM DIET, 40 HOURS POSTOPERATIVELY

| Procedure | No. of rats | Rate of urea N formation mg. N per 100 gms. body weight per hr. | | | | |
|---|---|---|---|---|---|---|
| Hours postoperatively | | 0–40 hrs. | "p" | 40–43 hrs. | "p" | 43–46 hrs. |
| Nephrectomized VuJ-n* (9.6 mg. N per 100 gm.) | 17 | 2.9±0.13 | N.S. | 3.2±0.14 | N.S. | 3.8±0.49 |
| "p" | | <0.01 | | <0.01 | | N.S. |
| Adrenalectomized nephrectomized VuJ-n* (9.6 mg. N per 100 gm.) | 15 | 2.3±0.14 | N.S. | 2.2±0.28 | <0.05 | 3.4±0.52 |
| Nephrectomized plasma* (10.4 mg. N per 100 gm.) | 8 | 2.3±0.07 | N.S. | 2.2±0.24 | <0.01 | 3.6±0.31† |
| "p" | | N.S. | | N.S. | | <0.01 |
| Adrenalectomized-nephrectomized plasma* (10.4 mg. N per 100 gm.) | 6 | 2.2±0.14 | N.S. | 1.9±0.61 | N.S. | 1.5±0.30 |
| Nephrectomized plasma* (10.4 mg. N per 100 gms. in 5% glucose | 7 | 2.3±0.12 | N.S. | 2.9±0.45 | <0.01 | 1.4±0.12† |
| "p" | | <0.01 | | <0.02 | | <0.01 |
| Adrenalectomized-nephrectomized plasma* (10.4 mg. N per 100 gms. in 5% glucose | 6 | 1.6±0.07 | N.S. | 1.2±0.41 | N.S. | 0.7±0.19 |

* Administered intravenously at the 43rd hour.
† "p" value <0.01 for the nephrectomized animals given plasma compared to the nephrectomized animals given plasma in 5% glucose.

response to this dose of glucose is consistent with previously reported results (Engel, Schiller and Pentz, 1949).

Table 2 records the results of experiments carried out 40–46 hours postoperatively. In this series the animals were usually quite ill during the experiment, so that the results obtained were not so uniform as in the 18 hour group.

After the injection of amino acids (VuJ-n) there was an increase in the rate of urea formation in the adrenalectomized animals. The increment, 1.2 mg. per 100 gm. per hour is comparable with 1.6 mg. per 100 gm. per hour observed 18 hours after operation. In animals with intact adrenals there was also an average increase in the rate of urea formation, but the rise was small (0.6 mg. per 100 gm. per hour) and not statistically significant. The lack of significance can be related in part to the large standard errors obtained in this series, when the animals were within a few hours of death. The larger response of the adrenalectomized animals may be due in part to the lower basal urea formation rate, and in part to the fact that adrenalectomized potassium-depleted animals develop uremia more slowly than do those with intact adrenals (Bondy and Engel, 1947) so that their condition was less desperate than that of the rats with intact adrenals at this time.

The injection of plasma was followed by no increase in the rate of urea formation in the animals without adrenals, but in those whose adrenals were intact, there was a rise which was comparable to that obtained at 18 hours, and considerably larger than that shown at 40 hours after the injection of VuJ-n. There was a significant difference in the rate of urea formation after the injection of plasma in the adrenalectomized rats as compared with those with intact adrenals. The response of the animals to VuJ-n and to plasma was similar at 18 and 40 hours postoperatively, suggesting that the effects of adrenalectomy are well established 18 hours after operation.

The extra urea formation when albumin was given to ACE treated rats was prevented if glucose was administered with the albumin (Engel, Schiller, and Pentz, 1949). It seemed of interest to determine whether the increase in urea after plasma which occurs in potassium deficient nephrectomized rats even in the absence of extra cortical hormone could be blocked by glucose. As seen in Table 2, the simultaneous administration of glucose and plasma produced a fall in the rate of urea formation in both adrenalectomized and non-adrenalectomized animals. The reduction in the rate of urea formation was significant in the animals with intact adrenals. It should be noted that glucose not only totally blocked the effect of the injection of plasma on the urea production rate of animals with intact adrenals but also reduced the higher rate of urea formation occurring at 40 hours as compared to 21 hours after nephrectomy. Thus the increases in urea formation after plasma and after the stress of increasing ure-

mia in rats with intact adrenals are subject to the same inhibitory influence from glucose as are those after excess ACE, with or without albumin.

## DISCUSSION

The response of the adrenalectomized rats to the administration of amino acids supports the point of view that the adrenal cortical hormones do not promote deamination of amino acids. It is apparent that, under the circumstances of the experiment, adrenalectomized rats were capable of deaminating amino acids as well as rats with intact adrenals. Evans (1941) had previously reported observations which agree with this finding. He showed that the injection of dl-alanine was followed by an increase of urea production in adrenalectomized-nephrectomized rats which was equal to that found in normal controls.

An explanation is not apparent for the increase in urea formation after plasma in the potassium-depleted nephrectomized rats compared to the lack of response to plasma or albumin in the presence of an adequate potassium intake. Conceivably it might be due to a decreased ability to store protein intracellularly in the face of a potassium deficiency. However, the possibility cannot be ruled out that, despite all precautions, this plasma used in these experiments was toxic and stimulated endogenous protein catabolism. In any case the failure of the adrenalectomized-nephrectomized rat to show an increase in urea formation after plasma unless cortical extract is given is significant. It indicates that the adrenal cortex is necessary for the increase in nitrogen metabolism that occurs after plasma although there is no proof here that it is responsible for it. This is similar to and may be even identical with, the relation of the adrenal cortex to the increased nitrogen excretion after stress. Ingle, Ward, and Kuizenga (1947) have shown that the adrenal cortex is necessary, but not responsible for this response. Our previous observation (Bondy and Engel, 1947) that the rate of urea formation in adrenalectomized rats remains constant from nephrectomy to death unless cortical extract is given, whereas it accelerates from day to day in rats with intact adrenals is another example of the role of the adrenal in the mobilization and catabolism of protein after stress. The increase in urea formation after plasma as well as the higher rate of urea formation 40 hours after nephrectomy were both modified by glucose just as was the stimulation of protein catabolism by adrenal cortical extract (Engel, Schiller, and Pentz, 1949). Since glucose had no effect on deamination of amino acids these results suggest that glucose is acting at the same level in all these cases, i.e. inhibition of protein breakdown itself.

When taken in conjunction with the normal rate of urea formation after amino acid injection in the adrenalectomized rats and the ob-

servations described in the previous report, the results with plasma are consistent with the view that the adrenal cortex intervenes in protein metabolism at the level of protein breakdown rather than elsewhere. Where there is the element of stress the action of the adrenal cortex in protein catabolism apparently is a secondary one and is linked to some other as yet unidentified factor.

## SUMMARY

The rates of urea formation in nephrectomized and adrenalectomized-nephrectomized rats previously on low potassium diets have been investigated as measures of nitrogen metabolism. Studies were conducted at 18 and 40 hours postoperatively.

The adrenalectomized-nephrectomized animals formed urea at the same rate as did the controls during the first 21 hours postoperatively but generally at a lower rate after 40 hours.

The adrenalectomized-nephrectomized rats formed urea at the same rate after an injection of amino acids as did the control nephrectomized rats.

The adrenalectomized-nephrectomized rats showed no increase in urea formation after injection of rat plasma, but did if adrenal cortical extract was given 2 and 3 hours before the plasma. The control nephrectomized rats responded to intravenous plasma injection by a significant increase in urea formation. The latter was prevented if glucose was given with the plasma.

Glucose intravenously had no effect on the rates of urea formation in either group of rats at 18 hours postoperatively. At 40 hours postoperatively it caused a significant decrease in urea formation in the nephrectomized rats with intact adrenals.

The data are interpreted to support the view that the action of the adrenal cortex in protein metabolism is at the level of protein break down itself rather than at the level of deamination of amino acids, regardless of whether this be a direct or an indirect action of the hormone.

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# NOTES AND COMMENTS

## THE THYROXINE-LIKE ACTION OF TETRABROM-THYRONINE

Abderhalden and Wertheimer (1928) and Gaddum (1929–30) have shown that 3:5:3′:5′-tetrabrom-thyronine, the bromine analogue of thyroxine, raises the metabolic rate of the rat when given in doses of twenty to fifty mgms. per kilo. It has now been shown that this substance in doses of 50 μg. per 100 gm. per day prevented the development of thyroid and pituitary changes in rats receiving methylthiouracil. The thyroids showed subnormal activity with completely flattened epithelium and colloid storage. The pituitary basophils were normal in appearance and number (8%). Tetrabrom-thyronine has, therefore, about one twentieth the biological activity of DL-thyroxine. As in the case of thyroxine, the amount necessary to prevent pituitary and thyroid changes is about one one hundredth of that necessary to produce a distinct increase in the metabolic rate.

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# NEW BOOKS

PREGNANCY DIAGNOSIS TESTS: A REVIEW. By Alfred T. Cowie. Published by the Commonwealth Agricultural Bureaux Joint Publication No. 13. Shinfield, Reading, Great Britain, 1948. 283 pp. 15s.

This is a most useful book. The author, of the National Institute for Research in Dairying, has searched the literature on pregnancy tests applicable to all species and has reviewed it in a most readable and interesting volume. Species differences plague the student of reproduction more vexatiously than in any other field of physiology. A sure-footed survey of the comparative aspects of even a part of this field is therefore welcome.

The various pregnancy tests, and they are legion, have been classed into seven main groups. They are the tests based on:

1. clinical methods
2. hormonal investigations of body fluids
3. enzymic investigations of body fluids
4. other biochemical investigations of body fluids
5. physiological phenomena
6. immunological phenomena
7. physical investigations of body fluids.

These chapters are rounded out by a brief but informative summary and conclusions. The bibliography contains nearly two thousand titles, and the book includes a well designed index.

Although, as is inevitable, woman receives her lion's share of attention, it is startling to find no mention of her in the chapter on clinical methods. It is true that such an omission is justified by the numerous textbooks of obstetrics that are available: nevertheless even a short account would have completed the section. Except for this one omission, the physiologist, the veterinarian and the physician alike will read the volume with interest and profit.

THE EPITHELIA OF WOMAN'S REPRODUCTIVE ORGANS. By George N. Papanicolaou, Herbert F. Traut and Andrew A. Marchetti. Published by the Commonwealth Fund, New York, 1948. vi+53 pp. Price $10.00.

The reproductive cycle of female mammals is accompanied by extensive changes in the morphology and physiology of the cells and tissues comprising the reproductive organs. The fact that cyclical variations exist has been recognized for long periods, but the accurate timing of the reproductive cycle, essential for any accurate study of the changes, became possible only during the last three decades. In 1917, Stockard and Papanicolaou recognized that the cytological study of vaginal smears provided an accurate end-point for studies of the reproductive cycle. This discovery ushered in a remarkable series of investigations clarifying the temporal sequences of the cycle in a number of mammalian species including man and leading directly to the further discoveries of the physiological effects and chemical natures of the ovarian hormones.

Despite the rapid increase in knowledge, the study of human reproduction has lagged behind that of the lower species. To a considerable extent, our concept of the human reproductive cycle represents inferences made by homology with the corresponding events as observed in animal experiments. Detailed information on the human species has been slow forthcoming. Studies such as those of Hertig and Rock on early human embryos, Papanicolaou on the human vaginal smear and now on the reproductive organs are therefore all the more welcome.

In the introduction, the authors state that:

"This monograph is not intended to take the place of a handbook. The interpretations given here largely reflect our own views, and some of them may be found to conflict with prevailing concepts. In our endeavor we have been guided by the desire to stimulate more thought in certain directions and to point out the growing importance of the cytologic approach."

Their account does indeed do violence to occasional preconceptions, especially those based on experiments on lower forms. Some species of monkeys have tough, fibrous adhesions of the ovary, induced by the prevailing filarial infections, which apparently do not hinder ovulation in the slightest. The present authors, on the contrary, state that a thick fibrous capsule hinders and prevents ovulation. The contents of the uterine lumen and the uterine glands may contain copious amounts of glycogen, according to the present study, but no attention is given to the possibility that this material may represent mucus, which spuriously gives many of the cytological reactions for glycogen. By analogy with the lower animals, mucus should be suspected in this location and recent investigations have proved its presence there in the human. The question remains, therefore, whether both glycogen and mucus occur in the secretions of the glandular lumina, or whether mucus alone can account for all of the observations.

The criticisms above suggest that in their zeal to present the most accurate and painstaking record of the cytology of the human reproductive tract, perhaps too little attention has been devoted to homologous situations. But this is a minor matter—the positive value of the detailed cytological information presented greatly outweighs the minor negative criticisms.

No review of this book could be complete without mention of the twenty-one plates of colored illustrations which document it. Some are semi-diagrammatic, representing histological interpretations, some are drawings and some are photomicrographs. All are carefully and beautifully printed. It is unfortunate that a few of the figures appear to be retouched without appropriate mention of the fact, and that plate 16 is mislabeled a photomicrograph when it is actually a drawing. Nevertheless, they represent the highest expression of the art of the histologist and the color-printer.

# ENDOCRINOLOGY

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# HORMONAL EFFECTS ON THE NUCLEIC ACID AND PHOSPHOLIPID TURNOVER OF RAT LIVER AND THYMUS[1]

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WITHIN the last decade pioneer work by Scandinavian investigators (Caspersson, 1940; Caspersson and Schultz, 1940; Euler and Hevesy, 1942; 1944; Andreasen and Ottensen, 1944; 1945; and Hevesy, 1946) has correlated the turnover of nucleic acids with the rate of growth of certain tissues. Similar findings have also been reported by Brues and his coworkers (1942; 1944), and by Marshak *et al.* (1941; 1945), working with isolated nuclei. The work of Claude (1943; 1944) has also shown the close connection with growth of nucleic acids and phospholipids in several relatively separate components of cytoplasm.

It is well known that hyperthyroid animals possess significantly heavier livers than do normal ones, that the administration of adrenal steroids and of pituitary adrenocorticotropic hormone causes a marked decrease in thymus weight, and that hypophysectomized animals do not gain weight whereas the same animals gain more than normal growing animals if treated with growth hormone. In view of the above findings, experiments were undertaken to ascertain the effects of the thyroid and adrenal hormones, and of pure growth hormone on the turnover of nucleic acids and phospholipids in the liver and thymus of rats.

## EXPERIMENTAL

### *Animals*

Group I) Approximately 200 g. female rats which had received stock diet containing 1% desiccated thyroid powder (Armour) for one month and untreated controls.

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TABLE 1. EFFECT OF VARIOUS HORMONES ON THE NUCLEIC ACID AND PHOSPHOLIPID TURNOVER IN LIVER AND THYMUS OF RATS

| Group | Treatment | Blood inorg. P S. A. | Liver nucleic acids | | Thymus nucleic acids | | Liver phospholipids | | Thymus phospholipids | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | S. A. | S. A. | S. A. | R. A. | S. A. | R. A. | S A. | R. A. |
| I | Normal (2)* | 0.96 | 0.082 | 0.085 | 0.106<br>(1) | 0.099†<br>(1) | 0.46 | 0.47 | 0.134 | 0.125†<br>(1) |
| | Thyroid Powder (2) | 0.46<br>(−52%) | 0.049<br>(−40%) | 0.106<br>(+25%) | 0.055<br>(−48%) (1) | 0.138†<br>(+39%) (1) | 0.13<br>(−22%) | 0.83<br>(+77%) | 0.023<br>(−83%) | 0.057†<br>(−54%) (1) |
| II | Normal (4) | 1.27 | 0.09 | 0.071 | 0.092 | 0.072 | 0.61 | 0.48 | 0.052 | 0.041 |
| | 17-dehydrocorticosterone (2) | 1.48<br>(+17%) | 0.10<br>(+11%) | 0.068<br>(−4%) | 0.116<br>(+26%) | 0.079<br>(+10%) | 0.45<br>(−26%) | 0.30<br>(−38%) | 0.070<br>(+36%) | 0.047<br>(+15%) |
| | Cortical Extract (2) | 1.12<br>(−11%) | 0.10<br>(+11%) | 0.089<br>(+25%) | 0.111<br>(+21%) | 0.099<br>(+38%) | 0.61 | 0.54<br>(+13%) | 0.051<br>(−2%) | 0.046<br>(+12%) |
| III | Normal (4) | 1.51<br>(3) | 0.15 | 0.99 | 0.110<br>(3) | 0.073 | 0.67 | 0.44 | 0.062<br>(3) | 0.041<br>(3) |
| | Hypophysectomy (3) | 2.98<br>(+97%) (2) | 0.13<br>(−13%) | 0.44<br>(−56%) | 0.069<br>(−37%) (2) | 0.023<br>(−68%) (2) | 0.38<br>(−43%) | 0.13<br>(−71%) | 0.027<br>(−56%) (2) | 0.009<br>(−78%) (2) |
| | Growth Hormone in Hypophysectomized Rats (3)‡ | 1.94<br>(−35%) (2) | 0.14<br>(+8%) | 0.72<br>(+64%) | 0.108<br>(+57%) (2) | 0.056<br>(+144%) | 0.55<br>(+45%) | 0.28<br>(+115%) | 0.063<br>(+133%) (2) | 0.033<br>(+267%) (2) |

* Figures in parentheses indicate number of rats; duplicate analyses were made of liver fractions, single analyses of thymus fractions.

† Based on R. A. of blood being 1.07 and 0.40 for normal and hyperthyroid animals respectively.

‡ Percentage differences expressed for values obtained with tissue from growth hormone treated hypophysectomized rats are based on values obtained with untreated hypophysectomized controls.

Group II) Approximately 90 g. female rats which had received a daily subcutaneous injection of a total of (a) 18 mg. 17-dehydrocorticosterone (Merck) over a period of 8 days or (b) 25 R. U. Cortical Extract (Upjohn) over a period of 11 days, and (c) untreated controls.

Group III) Approximately 75 g. female hypophysectomized rats which had received daily intraperitoneal injections of 0.170 mg. pure growth hormone per day for 10 days and untreated hypophysectomized as well as normal controls.

*Material*

Radioactive phosphorus ($P^{32}$)[3] was administered intraperitoneally as inorganic phosphate on an average of 1.5 $\mu$c. per 100 g. rat, 2 hours prior to sacrifice.

*Procedure*

The animals were anesthetized with ether; a blood sample was taken from the inferior vena cava for determination of the specific activity of free plasma phosphate; the liver and thymus were removed, weighed, and promptly immersed in cold saline (0°C.). Tissue samples were weighed on a Roller-Smith torsion balance, homogenized in cold 4% trichloroacetic acid and separated into phospholipid, nucleic acid, and residual protein phosphorus fractions according to the method of Schneider (1945). Duplicate samples were analyzed for each liver; whereas the whole thymus was required for each analysis. Radioactivity was determined with the Geiger-Müller counter and chemical determination of phosphorus done by the Sumner method (1944).

## RESULTS AND DISCUSSION

The results are given in terms of specific activity (S. A.) and relative activity (R. A.). Specific activity is defined as the ratio of the percentage of administered $P^{32}$ to mg. $P.^{31}$ The relative activity is defined as the ratio of the specific activity of the fraction in question to the specific activity of the free plasma phosphate. The results are summarized in Table 1.

In the present studies it has been found that the complete cessation of growth as brought about by hypophysectomy in a young animal, results in a relative decrease in the turnover of nucleic acids and phospholipids as compared with unoperated animals. Conversely, treatment with growth hormone has caused a suggestive increased turnover of these fractions. Significant responses were elicited in the thymus, which increased 74% in weight, compared with its hypophysectomized control; whereas the responses in the liver, which increased by only 30%, were not as great.

Smaller and less consistent effects were obtained in the studies conducted with other hormone. Hyperthyroidism which caused enlargement of the liver (69% increase) led to an increased turnover of

[3] Kindly supplied by the Radiation Laboratory, University of California, Berkeley.

nucleic acids and phopholipids of the liver and of thymus nucleic acids but a decrease in the thymus phospholipid turnover.[4]

Treatment with 17-dehydrocorticosterone, which occasioned a slight loss of body weight (6 g.) and more considerable loss in thymus weight (32%) and liver weight (18%), did not cause the anticipated decrease in either nucleic acid or phospholipid turnover. Perhaps if larger doses of this steroid had been administered, more marked responses might have been obtained.

Cortical Extract in contrast to the cortical steroid, caused an increase in body weight (45 g.) and in liver weight (36%), and only an insignificant decrease in thymus weight (12%). In the case of both liver and thymus a slight increase of nucleic acid and phospholipid turnover occurred.

Studies in which only total nucleic acids are estimated might seem of questionable value since desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have such diverse functions. In these preliminary studies however we have been able to show clear effects especially in the thymus of hypophysectomized animals with and without growth hormone. All of these results on total nucleic acid turnover are undoubtedly measurements primarily of RNA, since it has been shown that there is about four times as much RNA as DNA in tissues (Brues, Tracy and Cohn, 1944; Davidson and Waymouth, 1943), and that the turnover of RNA is always greater than that of DNA (Hammarsten and Hevesy, 1946).

## SUMMARY

Hypophysectomy was associated with a decreased turnover of both nucleic acids and phospholipids in the rat's thymus and of the phospholipids in rat liver, with less effect on liver nucleic acids.

Growth hormone administered to hypophysectomized animals caused an increased turnover of nucleic acids in the thymus and of phospholipids in both liver and thymus.

Thyroid treatment was accompanied by a decreased specific activity of both nucleic acids and phospholipids in both liver and thymus, an increased relative activity of the phospholipids of the liver but decreased relative activity of the phospholipids of the thymus. It caused an increased relative activity of the nucleic acids in both organs.

Both 17-dehydrocorticosterone and Cortical Extract led only to slight changes in the turnover of either substance in both organs.

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[4] The great difference in magnitude of the values for the S. A. and R. A. of the nucleic acids and phospholipids observed in the hyperthyroid animals was in agreement with similar differences observed in the S. A. and R. A. of adenosinetriphosphate values observed in previous experiments (D. M. Greenberg, J. Fraenkel-Conrat and M. B. Glendening, unpublished). There difference has been attributed to the dilution of injected $P^{32}$ by a great increase in plasma inorganic $P^{31}$.

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an hour later. Data in Table 2 indicate results obtained. Conclusions drawn from these data may be summarized as follows: (a) with increase in concentration of sulfuric acid the color also increases, but there is also a parallel color increase in the respective reagent blanks; (b) to avoid a too deeply colored blank 19 N $H_2SO_4$ appears to be favorable for nearly maximum blue color for DA; (c) heating for 3 minutes is the most favorable time period, less than that produces much less color with DA and more does not greatly increase the blue

TABLE 2. CHOICE OF CONCENTRATION OF SULFURIC ACID AND HEATING TIME AFTER ADDITION OF THE ACID

| $H_2SO_4$ N conc. | Heating | 25 $\mu$g DA* | 50 $\mu$g DA | 75 $\mu$g DA | 100 $\mu$g DA | Blank |
|---|---|---|---|---|---|---|
| | in min. | % T* | % T | % T | % T | % T |
| 13.22 | 3 (7) | $94^3$ | 90 | 85 | $81^2$ | 100 |
| 16.00 | 3 | 93 (92) | $85^1$ (83) | 80 (77) | $72^3$ (69) | 98 |
| 16.85 | 3 | 83 (82) | 70 ($68^2$) | $60^1$ ($58^2$) | $51^1$ ($49^2$) | 96 |
| 17.83 | 2 | 83 ($82^1$) | 72 (71) | $65^2$ ($63^1$) | 60 (58) | $97^2$ |
| 17.83 | 3 | 79 (79) | 64 (63) | 51 (50) | 43 (42) | 92 |
| 17.83 | 4 | $76^1$ (76) | 61 (61) | 49 (49) | 42 (42) | $93^1$ |
| 17.83 | 5 | 77 (78) | 62 (63) | 49 (49) | 40 (40) | 89 |
| 18.85 | 2 | 76 ($75^3$) | 61 (61) | 50 ($49^2$) | 42 ($41^2$) | $95^2$ |
| 18.85 | 3 | 71 (72) | 52 (53) | 41 (42) | 33 ($33^2$) | $90^2$ |
| 18.85 | 4 | $75^2$ (76) | 53 (54) | 43 (44) | 35 ($35^2$) | 86 |
| 18.85 | 5 | 73 ($74^1$) | 54 ($55^3$) | $41^2$ ($43^1$) | 34 ($35^2$) | $82^3$ |
| 19.89 | 3 | 70 (71) | 56 (57) | $39^3$ (40) | $31^2$ (32) | 87 |
| 20.94 | 3 | $65^3$ (66) | | $36^3$ (37) | $28^1$ ($28^3$) | $86^2$, 88 |
| 21.67 | 3 | 68 (69) | 51 ($51^2$) | $37^3$ (38) | $29^3$ (30) | $79^2$ |
| 23.14 | 3 | 65 (65) | 47 (47) | 33 (33) | 27 (27) | 77 |

* DA = dehydroisoandrosterone; T = % transmission in an Evelyn colorimeter with filter 660. Columns under % T are first reading and in ( ) readings after an hour. The readings are all with the reagent blank, with sulfuric acid of the concentration for a given horizontal column, set at 100. The last column is a comparison of the reagent blanks against a reagent blank with 13.22 N $H_2SO_4$ set at 100.

color, while the color of the blank is appreciably deepened. In subsequent series of tests employing 19 N $H_2SO_4$ and read against blanks without furfural set at 100, the reagent blank readings varied between extremes of 88 and 93 with an average of 90 for 10 seperate determinations.

Method adopted for quantitative determination of DA: An alcoholic aliquot in a test tube containing DA within the range of 10–100 $\mu$g. is evaporated in a hot water bath and dried over $CaCl_2$ in a vacuum desiccator. To each tube, including blanks, 3.5 cc. of 60% acetic is added, which is then heated in a boiling water bath to bring the tube to the temperature of the bath, then add 1 cc. of 0.9% furfural solution, mix, heat in the boiling water bath for 5 minutes. Then add 6 cc. of 19 N $H_2SO_4$, mix, and heat for 3 minutes in the boiling water bath. Cool at once in an ice bath. Transfer to Evelyn tubes and read in an Evelyn colorimeter with filter 660 in 30 to 60 minutes after the final heating.

No special reagents are required except redistilled furfural. Fur-

fural solution was prepared fresh on the day it was used. However, furfural solutions kept refrigerated for a number of days showed no greater variations with different aliqouts of DA than did similar aliquots with freshly prepared furfural solutions. Baker's C.P. glacial acetic acid was employed for preparation of 60% acetic acid (60 cc. of glacial acetic and 40 cc. of distilled water). Baker's C.P. $H_2SO_4$ was used to prepare 19 N solution of the acid (50–50 by volume with distilled water, with subsequent adjustment to make the solution 19 N within 0.1N) .

Spectrophotometric adsorption curve: The adsorption curve of the color produced with DA by the indicated procedure was determined with a Beckmann spectrophotometer over the range of 325 to 900 mμ. With density plotted as ordinate for the mean of 2 determinations with 100 μg. DA, against the wave length in mμ. employed as abscissa, 2 maxima occur, one at 656 mμ. and the other, much lower value, at 370 mμ. There is also a slight plateau on the ascending curve from 610–620 mμ. Thus, photometrically the highest color value is obtained at 656 mμ., which confirms the optimum value obtained with filter 660 in the Evelyn colorimeter.

In subsequent data the 660 filter was employed with the Evelyn colorimeter and evaluations were made on the basis of the values of standard solutions of DA determined along with unknowns. All standards and unknowns were made up in absolute alcohol solution and suitable aliquots were evaporated to dryness in a hot water bath and dried further over $CaCl_2$ in vacuo, followed by the procedure indicated above.

Method of calculation of DA in specimens: Calculations were made on the basis of the mean color density per μg. of DA. A reagent blank served as control in case of pure solutions. In case of aliquot of unknowns a specimen blank and a reagent blank were obtained against a zero blank, i.e., a blank containing water in place of furfural, to make proper allowance for the non-specific colors in the unknown and in the reagent blank. Thus, for the determination of DA in an unknown, the following set of tubes is employed: (1) zero blank, reagents with water in place of furfural, read ultimately at 100 with filter 660 in the Evelyn Colorimeter; (2) reagent blank, for non specific color value of reagents with furfural; (3) a series of tubes with DA within the limits of 10–75 μg.; (4) an aliquot of unknown without furfural, for the non specific color in the unknown; and (5) an aliquot of unknown, as in tube (4), with furfural. The net color density for the standard DA tubes is obtained by subtraction of the value in (2) from that in each of the tubes in (3). Dividing the result by the μg. employed gives the density per μg. of DA. The mean value for the series of standards is then calculated. The net color density value for the unknown is obtained by subtracting the sum of densities in (2) and (4) from the value in (5). Dividing this value by the

mean color density per $\mu$g. of DA then gives the $\mu$g. of DA in the aliquot of the unknown. Since the specimen blank is usually small, procedure and calculations may be simplified in most cases by omitting the zero blank and the specimen blank and setting the reagent blank at 100 on the galvanometer scale. This introduces but a small error in most cases.

Table 3 presents data obtained with many determinations of aliquots of DA ranging from 10–150 $\mu$g. The weighted mean density value is calculated in accordance with the density and number of determinations employed for each of the various aliquots. A curve plotted on the basis of the calculated density values for the range 10–100 $\mu$g. DA (column 4, Table 3) and employed for placing mean

TABLE 3. DEHYDROISOANDROSTERONE (DA) DENSITY VALUES OF RANGE 10–150 $\mu$g.

| DA $\mu$g. | Observed density mean value | Density per $\mu$g. $\times 10^5$ | Density calculated |
|---|---|---|---|
| 10 | 0.0530 (3) | 530 | 0.0492 |
| 15 | 0.0744 (4) | 496 | 0.0738 |
| 20 | 0.1001 (3) | 500.5 | 0.0984 |
| 25 | 0.1288 (12) | 515 | 0.1231 |
| 50 | 0.2480 (11) | 496 | 0.2461 |
| 65 | 0.3470 (1) | 533.8 | 0.3199 |
| 75 | 0.3584 (9) | 477.9 | 0.3692 |
| 90 | 0.4690 (1) | 421.1 | 0.4430 |
| 100 | 0.4580 (12) | 458 | 0.4922 |
| 125 | 0.5270 (3) | 421.4 | — |
| 150 | 0.6580 (1) | 438.7 | — |

Weighted mean density 492.2 (10–100 $\mu$g.)

The number in ( ) in the second column indicates the number of determinations employed. Density $= 2 - \log \cdot G$, where G is the galvanometer reading with the reagent blank set at 100 and read with filter 660 in an Evelyn colorimeter.

values of individual determinations (column 2, Table 3) and other single determinations of DA indicated that points for 10 to about 70 $\mu$g. of DA lie close to the straight line curve, but points for values above this range deviate more and more and fall below the line. Thus, the color is reasonably valid for measurements in the range of 10–70 $\mu$g. of DA. However, since small variations do occur, even though all conditions are strictly adhered to, a set of standards has been used with all determinations, instead of relying in a general calibration curve.

Determination of DA in presence of androsterone: Solutions of mixtures of these 2 steroids in various ratios and absolute amounts were estimated for DA values by the procedure as indicated. Table 4 indicates the results obtained. It may be noted that the values tend to be low when large aliquots are mixed with androsterone, but large aliquots should be avoided in any case.

Specificity of the reaction with steroids of various types: A comparison of 100 $\mu$g. aliquots of substances which produced a positive color is indicated in Table 5. All of the steroids tested may be con-

TABLE 4. DETERMINATION OF DEHYDROISOANDROSTERONE (DA) IN THE PRESENCE OF ANDROSTERONE (A)

| DA $\mu$g. | A $\mu$g. | Ratio DA/A | Observed density* | Observed density / Density of DA |
|---|---|---|---|---|
| 25 | | | 0.1308 | 1.00 |
| 25 | 37.5 | 0.67 | 0.1367 | 1.04 |
| 25 | 50 | 0.5 | 0.1235 | 0.945 |
| 25 | 100 | 0.25 | 0.1295 | 0.99 |
| 50 | | | 0.2347 | 1.00 |
| 50 | 25 | 2.0 | 0.2339 | 0.995 |
| 50 | 50 | 1.0 | 0.2366 | 1.01 |
| 50 | 75 | 0.67 | 0.2518 | 1.07 |
| 50 | 100 | 0.5 | 0.2666 | 1.13 |
| 100 | | | 0.4800 | 1.00 |
| 100 | 25 | 4.0 | 0.4395 | 0.915 |
| 100 | 50 | 2.0 | 0.4545 | 0.945 |
| 100 | 100 | 1.0 | 0.4255 | 0.885 |

* Density = 2 − log · G, where G is the reading of the galvanometer. All readings were made with the reagent blank set at 100 with filter 660 in an Evelyn colorimeter.

veniently grouped according to general structure and carbon content. These substances were all tested in 100 $\mu$g. aliquots, and in some instances in amounts up to 1 and 5 mg. Except where followed by a (+) they were all similar to the reagent blank. Androstane type, C19: androsterone, isoandrosterone (+), androstanediol-3$\beta$, 17$\alpha$, androstenediol-$\Delta^5$-3$\beta$, 17$\alpha$ (+), 17-methyl-androstanediol-3$\beta$, 17$\alpha$ (+), 17-methyl-$\Delta^5$-3$\beta$, 17$\alpha$ -androstanediol (+), 17-ethyl-$\Delta^5$-3$\beta$, 17$\alpha$ -androstenediol (+), androstenedione, dehydroisoandrosterone (+), dehydroisoandrosterone acetate (color value equal to the steroid content unesterified), testosterone, cis-testosterone (+), testosterone propionate, methyl testosterone, ethinyl testosterone. Estrane type, $C_{18}$: estrone, $\alpha$-estradiol, $\alpha$-estradiol benzoate, $\alpha$-estradiol dipropionate, ethinyl-$\alpha$-estradiol. $C_{21}$ type: progesterone, desoxycorticosterone acetate, pregnenolone (+), $C_{24}$ type, bile acids: cholic acid (+), desoxycholic acid, lithocholic acid, 3, 12-dihydroxy-7-ketocholanic

TABLE 5. POSITIVE COLOR AND DENSITY OBTAINED WITH VARIOUS STEROIDS

| Substance | Color | Color density* |
|---|---|---|
| Cholic acid | blue | 0.2596 |
| Dehydroisoandrosterone | blue | 0.4140 |
| Androstenediol-$\Delta^5$,3$\beta$,17$\alpha$ | blue, purplish tint | 0.4090 |
| Pregnenolone | blue with green tint | 0.2366 |
| Isoandrosterone | faintly blue | 0.0593 |
| Cis-testosterone | blue, dark hue | 0.0809 |
| 17-methyl-$\Delta^5$-androstenediol-3$\beta$,17$\alpha$ | purplish tint | 0.0731 |
| 17-ethyl-$\Delta^5$-androstenediol-3$\beta$,17$\alpha$ | purplish-pink | 0.1065 |
| 17-methyl-androstanediol-3$\beta$,17$\alpha$ | faintly blue | 0.0302 |

* Density = 2 − log · G, where G is the galvanometer reading. All density values are the mean of 2 determinations with 100 $\mu$g. of substance. Readings were made with the reagent blank set at 100 and with filter 660.

TABLE 6. ESTIMATION OF DEHYDROISOANDROSTERONE IN A VARIETY OF URINARY EXTRACTS

| Specimen | Aliquot used cc. | DA found in aliquot μg. | Da found* per cc. mg. | Da found in total specimen mg. |
|---|---|---|---|---|
| β Fraction, pooled, volume 10 cc. | 0.10<br>0.15 | 33.20<br>47.41 | 0.332<br>0.316 | |
| 5β, volume 5 cc., dilution 1-3 | 0.05<br>0.10 | 36.59<br>72.69 | 2.195<br>2.181 | 10.977<br>10.904 |
| B-1126, before fractionation, vol. 10 cc. diln. 1-10 | 0.10<br>0.20 | 10.21<br>20.97 | 1.021<br>1.049 | 10.210<br>10.485 |
| S-, pooled β fractions, dilution 1-25 | 0.10<br>0.20 | 31.84<br>70.08 | 7.96<br>8.76 | |
| B-749, before fractionation, vol. 10 cc., diln. 1-10 | 0.05<br>0.10 | 59.29<br>102.9 | 11.858<br>10.29 | 118.58<br>102.9 |

* DA = dehydroisoandrosterone, calculated on the basis of a zero blank and with a set of pure DA solutions as standards.

acid, 3, 7-dihydroxy-12-ketocholanic acid, 3, 6-diketo cholanic acid, 3, 12-diketo cholanic acid, 3-keto-6-hydroxy-cholanic acid, 3, 7-diketo cholanic acid, 3-hydroxy-7, 12-diketo cholanic acid. $C_{27}$: cholesterol. . . . Some of the substances other than those indicated by (+) produce a slight yellow color when aliquots above 500 μg. and up to 2 mg. are employed. In case of cholesterol with 1 and 2 mg. aliquots the % transmission was 96 and 94, respectively, with filter 620.

Estimation of DA in urinary extracts: Examples are presented in

TABLE 7. RECOVERY OF DA ADDED TO VARIOUS URINARY EXTRACTS

| Specimen* | Aliquot used cc. | DA added μg. | DA found μg. | DA recovered μg. | DA recovered % |
|---|---|---|---|---|---|
| *Before fractionation* | | | | | |
| B-1126, 10 cc.<br>dilution 1-10 | 0.20<br>0.20 | <br>25 | 20.97<br>46.38 | <br>25.41 | <br>101.6 |
| B-1149, 10 cc.<br>dilution 1-10 | 0.20<br>0.20 | <br>25 | 25.37<br>49.74 | <br>24.37 | <br>97.5 |
| *Ketone fractions* | | | | | |
| B-569, 5 cc.<br>dilution 1-25 | 0.10<br>0.10 | <br>25 | 40.57<br>66.48 | <br>25.91 | <br>103.6 |
| E-, pooled<br>(deep red color) | 0.10<br>0.10 | <br>25 | 19.61<br>43.08 | <br>23.47 | <br>93.9 |
| *β fractions* | | | | | |
| 11β, 5 cc.<br>dilution 1-3 | 0.10<br>0.10 | <br>25 | 69.21<br>93.99 | <br>24.78 | <br>99.1 |
| 17β, 5 cc.<br>dilution 1-3 | 0.10<br>0.10 | <br>25 | 55.63<br>82.79 | <br>27.16 | <br>108.6 |

* The specimens are urine extracts from adrenal tumor cases. DA = dehydroisoandrosterone.

Table 6. The values obtained with different aliquots check quite well in the first 3 examples, but in the last 2 examples they are not so satisfactory. In the last example, however, the lower value was to be expected on the basis of the high content in the larger aliquot.

Recovery of DA added to urinary extracts: Data are presented in Table 7. Two examples are given for each of 3 types of extracts, (a) neutral steroids before fractionation, (b) ketone fractions of neutral steroids, (c) beta fractions of digitonin fractionations of ketone fractions. The specimens employed are urine extracts from adrenal tumor cases.

TABLE 8. COMPARISON OF VARIOUS METHODS FOR THE DETERMINATION OF DEHYDROISOANDROSTERONE IN $\beta$ FRACTIONS

| Method† | Specimens* | | | | | |
|---|---|---|---|---|---|---|
| | A mg. DA | B mg. DA | C mg. DA | D mg. DA | E mg. DA | F mg. DA |
| 1 | 11.289 | 12.238 | 12.565 | 12.421 | 7.913 | 9.404 |
| 2 | 11.325 | 12.390 | 12.0 | 12.0 | 11.325 | 12.15 |
| 3 | 10.70 | 11.140 | 10.615 | 11.35 | 7.465 | 9.20 |
| 4 | 11.232 | 10.997 | 12.192 | 11.853 | 8.35 | 9.408 |

* The specimens represent pairs of digitonin fractionations of ketone fractions from urinary extracts of 3 adrenal tumor cases. The duplicate pairs are A and B, C and D, and E and F, each represents $\frac{1}{3}$ of the total ketone fraction of a 24 hr. urine extract. A part of specimen E was lost in the manipulation.

† Method, Holthorff and Koch's adaptation (1940) of Zimmermann's method for ketosteroids (1935) slightly modified by the author (1943); method 2 is the method of Pincus (1943) employing $SbCl_3$ as the color reagent; method 3 is specific for dehydroisoandrosterone, developed by the author (1948); method 4 is the present method.

Comparison of various methods for the determination of DA in beta fractions: Results are indicated in Table 8 for DA determined in urinary extracts by 4 different methods. Only beta fractions could be employed, since the other methods could be applied only to such fractions for direct comparison.

## DISCUSSION

The data here presented have indicated that estimation of pure DA can be done with reasonable precision by this modified Pettenkofer reaction. Further, the presence of androsterone does not interfere appreciably in this estimation within the working range of the method. In applying the procedure to urinary extracts added DA is nearly completely recovered from such extracts, whether they be unfractionated, ketone fraction or $\beta$ fraction. Finally, comparison of determination of DA in fractions of urinary extracts by this procedure with several other methods indicates reasonably close agreement. The method may, therefore, be employed for reasonably precise determinations of DA in urinary fractions. It may also be used in preliminary analyses of unfractionated and ketone fractionated ex-

tracts. Munson *et al.*[3] (1948) have developed the analysis for DA, based on the Pettenkofer reaction. Results obtained by the method here presented appear to be similar qualitatively and quantitatively, but this method has the advantage of being simpler in technic.

While analyses for DA in pure solutions and in various urinary extracts seem reasonably satisfactory, comparison with other steroids indicates that the specificity is far from being unique for DA. The data presented here on specificity is in substantial agreement with the interpretations of Munson *et al.* (1948) and with the data of Kerr and Hoehn (1944). Certain structural features of the steroid nucleus appear to be required for a positive reaction. The most important features appear to be $\Delta^5$ unsaturation, along with color enhancing, $\beta$-directed -OH (or other univalent group) in $C_3$ position. Good examples are DA; $\Delta^5$-androstenediol-3$\beta$, 17$\alpha$; $\Delta^5$-pregnenol-3$\beta$-one-20; and 3-hydroxy-$\Delta^5$-cholenic acid (lithocholenic acid). Saturation on $C_5$, as in isoandrosterone, greatly reduces the color, while a simultaneous saturation on $C_5$ and reversal of the -OH on $C_3$, as in androsterone, abolishes the color completely. Retention of unsaturation on $C_5$ and removal of the functional group on $C_3$, as in $\Delta^{4,5}$-androstanedione-17, reduces the color only in a small degree. However, certain anomalies appear. Thus, cholesterol, which has the above specified specific conditions gives no color, and cholic acid, in which the specific conditions are absent gives the color. Substitution of hydrogen on $C_{17}$ is also of some significance, since alkyl substitution greatly diminishes the chromogenicity. Oxidation of secondary alcohol groups in cholic acid and chenodesoxycholic acid to the corresponding dehydro acids also abolishes the color, indicating importance of structural features of the steroid nucleus other than on Carbons 3, 5 and 17.

It was hoped that this colorimetric method might be sufficiently specific so that $\beta$ fractionation or even ketone fractionation might be unnecessary. This hope, however, is not realized, for $\alpha$ fractions of specimens hitherto tested all contained more or less chromogenic substance qualitatively the same as for DA, and in some unfractionated urine extracts the color equivalent, calculated as DA, represented a larger amount of DA for the total specimen than was found by the Zimmermann technic in the same specimens calculated as androsterone (A). Obviously, in the unfractionated specimen substances other than DA or A are indicated by this procedure. Likewise in the $\alpha$ fraction, hence also in the ketone fraction prior to digitonin fractionation, substances other than DA are also indicated. In the $\beta$ fraction, as has been shown, determination of DA in various specimens by comparative methods indicated nearly identical results by

[3] This report first came to the author's attention when the work here presented was virtually completed.

4 different methods. Nevertheless, even here substances similar to DA chromogenically cannot be entirely excluded.

Finally, these various specificity aspects may be of use in indiacting or excluding the presence of substances which possess structural features chromogenically favorable in this reaction.

## SUMMARY

A colorimetric method has been developed for the quantitative estimation of dehydroisoandrosterone, applicable to pure solutions and to urinary neutral steroid extracts.

The method is based on a modified Pettenkofer technic, employing a boiling water bath as source of heating, an acetic acid solution with addition of furfural for the initial reaction, and addition of sulfuric acid for the final color development.

Specificity is discussed on the basis of tests on a variety of different types of steroids as reported here and as indicated in reports elsewhere. Specificity is not unique for dehydroisoandrosterone. In general, it is indicative of $\beta$ OH on $C_3$ and unsaturation on $C_5$, but is not conclusive.

Application of the method to quantitative estimation of dehydroisoandrosterone in fractions of urinary extracts is reasonably satisfactory. Limitations of the application to unfractionated specimens or ketone fractions are discussed. Use of the method to indicate presence or absence of substances with favorable chromogenic structural features is suggested.

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# THE RELATIVE GROWTH OF THE THYROID GLAND IN THE BOVINE FETUS[1]

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THE NATURE of thyroid iodine in the postnatal state is fairly well understood. At least 90 per cent of it is organic and almost all of this can be accounted for by two fractions: thyroxine-like and diiodotyrosine-like iodine.[3] These two compounds do not exist in the free state in the gland but are combined there with other amino acids to form the characteristic thyroid protein, thyroglobulin.

The nature of the iodine in the fetal organism has not been clearly established. To obtain such information, a study of various iodine fractions of the bovine fetal thyroid was undertaken. Data on that phase of the study will be presented in the following paper. The material acquired during these experiments also provided an opportunity for studying the growth of the gland in relation to body weight, body length, and age of the bovine fetus from 62 days to term (278–285 days depending upon breed). The present paper is concerned with this phase of the study.

## EXPERIMENTAL

Intact uteri of pregnant beef were collected randomly at abattoirs at the time of slaughter. The embryos and fetuses were weighed and measured immediately after the umbical cord was tied off at the fetal level. The thyroids were then removed, freed of extraneous connective tissue and fat, and weighed. A small portion was fixed in Bouin's solution for histological examination.

The fetuses were coellcted from random herds. Breeding or stock histories were not available. The present series comprises a total of 121 bovine fetuses obtained from dams chiefly of the Hereford breed (97 specimens). Four fetuses were obtained from Guernsey, 15 from Holstein, and five from Jersey breeds. Since the slopes of curves relating thyroid weight to body weight did not differ for the two sexes, no distinction was made between male and female fetuses.

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[2] U. S. Public Health Fellow.

[3] Monoiodotyrosine has recently been found in the thyroid gland by Fink *et al.* (1948) and by Taurog *et al.* (1949).

## RESULTS

In order to determine fetal ages, we employed the data of Winters *et al.* (1942) who measured body dimensions of fetal calves of known ages. In the present study, the age of each fetus was taken as the average of ages derived from the following four measurements: body weight, crown-rump (C.R.) length, chest circumference, and abdominal circumference. These were determined by methods previously described (Nichols, 1944).

The ages of the fetuses ranged from 62 days to term. In table 1, they have been grouped with respect to arbitrarily selected age ranges and the body measurements have been recorded for each range.

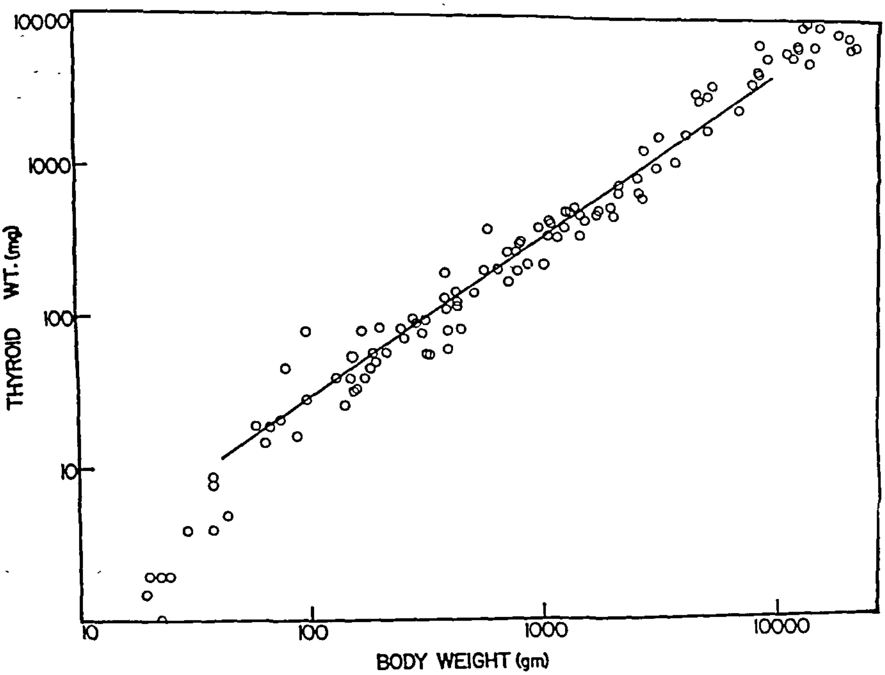


FIG. 1. Double-logarithmic plot of fetal thyroid weight against body weight.

When thyroid weights were plotted on a double logarithmic grid against total body weight (fig. 1), C. R. length (fig. 2), and calculated age (fig. 3), straight-line curves were obtained.

The data shown in figs. 1–3 were therefore fitted to the simple allometry equation of Huxley (1932):

$$y = bx^k \tag{1}$$

where $y$ = thyroid weight expressed in mg.

$x$ = body weight expressed in gm. (fig. 1), or C.R. length in mm. (fig. 2), or age in days (fig. 3).

TABLE 1. THYROID WEIGHTS AND BODY DIMENSIONS OF FETAL CALVES FROM 62 DAYS TO TERM

| Calculated age | No. of fetuses | Body weight | | C.R. length | | Chest circum. | | Abdom. circum. | | Thyroid weight | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Range | Mean | Range | Mean | Range | Mean | Range | Mean | Range | Mean |
| days | | gm. | gm. | mm. | mm. | mm. | mm. | mm. | mm. | mg. | mg. |
| 62–70 | 9 | 18–34 | 27 | 73– 96 | 85 | 51– 75 | 66 | 61– 80 | 70 | 1–9 | 4 |
| 75–85 | 10 | 42–150 | 84 | 104–153 | 127 | 87–120 | 99 | 82–122 | 100 | 5–45 | 25 |
| 87–95 | 14 | 96–235 | 168 | 151–185 | 165 | 115–133 | 123 | 115–149 | 129 | 24– 82 | 53 |
| 98–105 | 16 | 240–433 | 350 | 178–222 | 203 | 142–168 | 154 | 144–174 | 161 | 57–183 | 90 |
| 106–120 | 15 | 433–860 | 639 | 215–273 | 245 | 167–208 | 189 | 175–214 | 183 | 117–348 | 200 |
| 125–135 | 11 | $0.98 \cdot 10^3$–$1.50 \cdot 10^3$ | $1.21 \cdot 10^3$ | 295–334 | 312 | 214–253 | 233 | 227–265 | 252 | 202–471 | 350 |
| 136–150 | 9 | $1.52 \cdot 10^3$–$3.13 \cdot 10^3$ | $1.94 \cdot 10^3$ | 325–418 | 353 | 243–352 | 276 | 270–354 | 295 | $0.32 \cdot 10^3$–$1.37 \cdot 10^3$ | 542 |
| 152–160 | 9 | $2.10 \cdot 10^3$–$3.82 \cdot 10^3$ | $2.76 \cdot 10^3$ | 382–462 | 408 | 280–331 | 301 | 282–342 | 320 | $0.42 \cdot 10^3$–$1.10 \cdot 10^3$ | 709 |
| 163–175 | 7 | $4.34 \cdot 10^3$–$5.60 \cdot 10^3$ | $4.95 \cdot 10^3$ | 445–590 | 511 | 349–435 | 381 | 367–453 | 394 | $1.13 \cdot 10^3$–$2.74 \cdot 10^3$ | $2.01 \cdot 10^3$ |
| 190–210 | 9 | $7.25 \cdot 10^3$–$13.0 \cdot 10^3$ | $9.83 \cdot 10^3$ | 573–703 | 622 | 408–508 | 461 | 428–564 | 486 | $2.85 \cdot 10^3$–$6.69 \cdot 10^3$ | $4.01 \cdot 10^3$ |
| 213–228 | 5 | $12.5 \cdot 10^3$–$15.7 \cdot 10^3$ | $14.5 \cdot 10^3$ | 700–735 | 710 | 535–562 | 548 | 532–597 | 575 | $3.91 \cdot 10^3$–$6.95 \cdot 10^3$ | $5.40 \cdot 10^3$ |
| 240–260 | 7 | $19.9 \cdot 10^3$–$32.5 \cdot 10^3$ | $24.7 \cdot 10^3$ | 790–855 | 838 | 617–676 | 650 | 645–705 | 673 | $4.59 \cdot 10^3$–$11.4 \cdot 10^3$ | $6.54 \cdot 10^3$ |

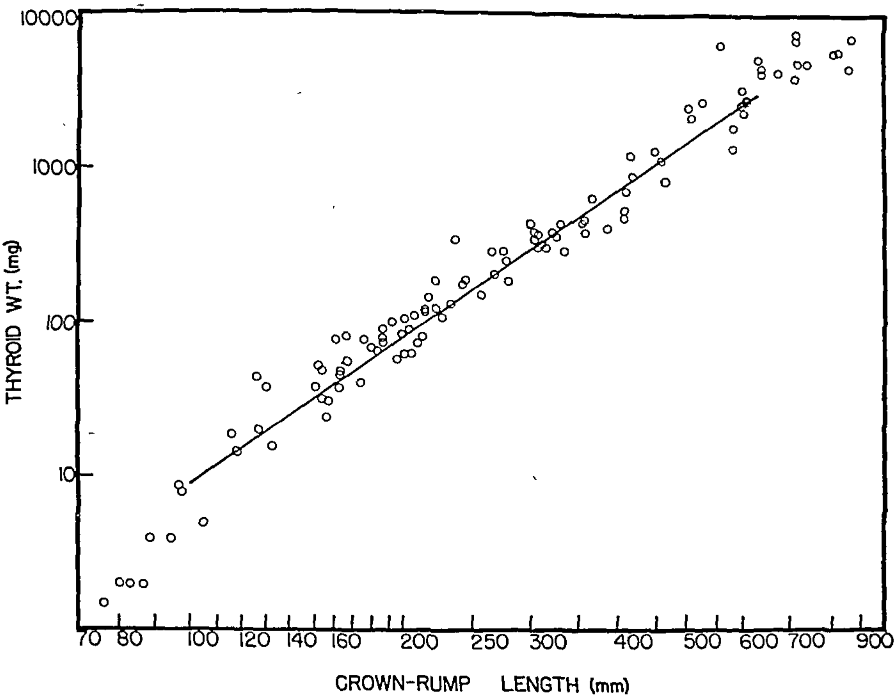


FIG. 2. Double-logarithmic plot of fetal thyroid weight against crown-rump length.

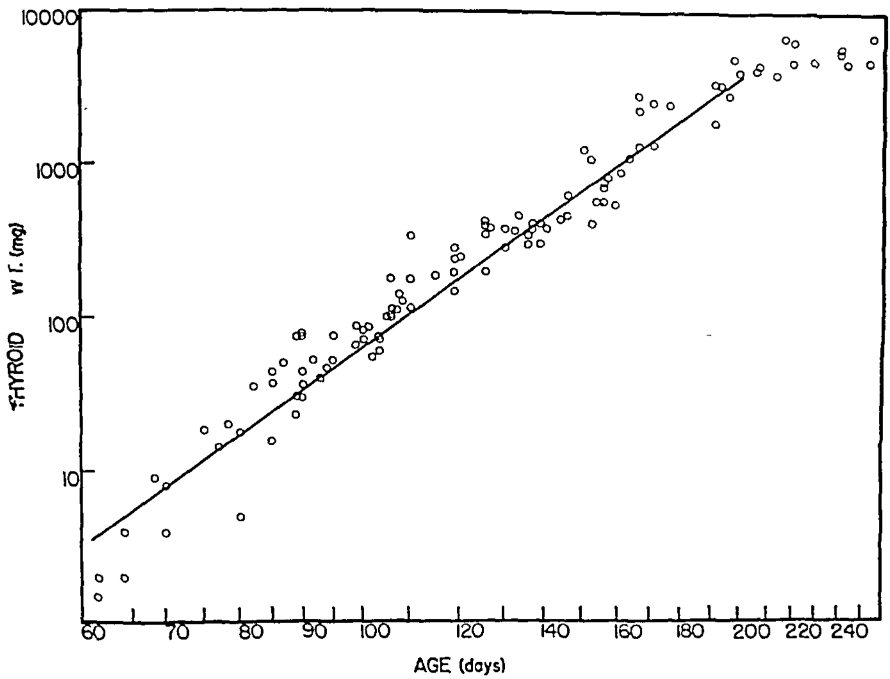


FIG. 3. Double-logarithmic plot of fetal thyroid weight against calculated age.

$b$ (the initial growth index of Reeve and Huxley, 1945)
= a constant that indicates the value of $y$ when $x$ is 1, i.e., a scale factor.

$k$ = the allometric growth constant.

*Relation to Body Weight*

In the case of fig. 1, equation (1) becomes

$$y = 0.25x^{1.0} \tag{2}$$

Equation (2) is valid from approximately 40 to 10,000 gm. body weight and from 11 to 3000 mg. thyroid weight.

The relative growth constant of the fetal thyroid with respect to body weight is 1.0. This indicates that during prenatal life, thyroid weight is almost directly proportional to body weight.

It is of interest to note that our value for $k$, namely 1.0, which is based on 121 measurements, is in good agreement with values reported earlier by Abeloos (1946) for calf fetuses (0.95), and by Schultze and Turner (1945) for fetal goat thyroids (0.98). Brody and Kibler (1941), furthermore, found a value of 0.924 for the relation of thyroid weight to body weight in a large variety of mature mammals.

*Relation to Body Length*

The expression for the curve shown in fig. 2 (thyroid weight plotted against C.R. length) is

$$y = (4.6 \cdot 10^{-6})x^{3.4} \tag{3}$$

The validity of this formula extends from 100 to 600 mm. C.R. length and from 10 to 4000 mg. thyroid weight.

*Relation to Age*

The curve representing thyroid growth in relation to age is shown in fig. 3. The following formula was found to fit the observed data:

$$y = (2.3 \cdot 10^{-11})x^{6.2} \tag{4}$$

This formula holds from 60 to 200 days of age with a corresponding thyroid weight range of from 2.7 to 4800 mg. Beyond 200 days of age there was considerable spread in the data.

The manner in which the values for the constants $k$ and $b$ were derived is shown below for fig. 3.

Let $x_1 = 60$ days and $x_2 = 200$ days; then $y_1$ (thyroid weight) = 2.7 mg. and $y_2 = 4800$ mg.
Therefore,

$$k = \frac{\log_{10} y_2 - \log_{10} y_1}{\log_{10} x_2 - \log_{10} x_1}$$

$$= \frac{\log_{10} 4800 - \log_{10} 2.7}{\log_{10} 200 - \log_{10} 60}$$

$$= 6.2.$$

The value for $b$ was calculated from the expression $\log b = \log y_0 - k \log x_0$, where $y_0$ (organ weight) and $x_0$ (age in days) are values on the plot of thyroid weight against age from approximately 62 to 200 days. For example, at 200 days:

$$\begin{aligned} \log_{10} b &= \log_{10} y_0 - k \log_{10} x_0 \\ &= \log_{10} 4800 - 6.2 \log_{10} 200 \\ &= 2.3 \times 10^{-11} \end{aligned}$$

*Percentage Growth Rate*

The percentage growth rate of an organ can be obtained from the expression

$$\frac{d \text{ (organ weight)}/d \text{ (age)}}{\text{organ weight}}.$$

Employing the above notation, this expression becomes

$$\frac{dy/dx}{y}.$$

If the absolute rate of growth of the thyroid gland is $dy/dx$, then

$$\begin{aligned} \frac{d}{dx}(y) &= \frac{d}{dx}(bx^k) \\ &= bkx^{k-1} \\ &= bx^k(kx^{-1}). \end{aligned}$$

Therefore

$$\frac{dy}{dx} = y\frac{k}{x}$$

and

$$\frac{dy/dx}{y} = \frac{k}{x}$$

For example, in the calf at the age of 75 days, $[(dy/dx)/y] = (k/x) = (6.2/75) = 0.083$. Therefore, the rate of growth of the fetal calf thyroid at 75 days is 8.3 per cent per day, i.e., per cent of its weight at this age.

Fig. 4 shows that the percentage growth rate declines steadily with increasing age. Thus, at 60 days, the percentage growth rate (instantaneous relative growth rate $\times 100$) is 10.2 per cent per day; at 220 days, approximately 2.8 per cent per day. Such a decline has also been demonstrated by Lowrey (1911) for the fetal pig thyroid and by Fenger (1913) for the fetal calf thyroid. The curve in fig. 4

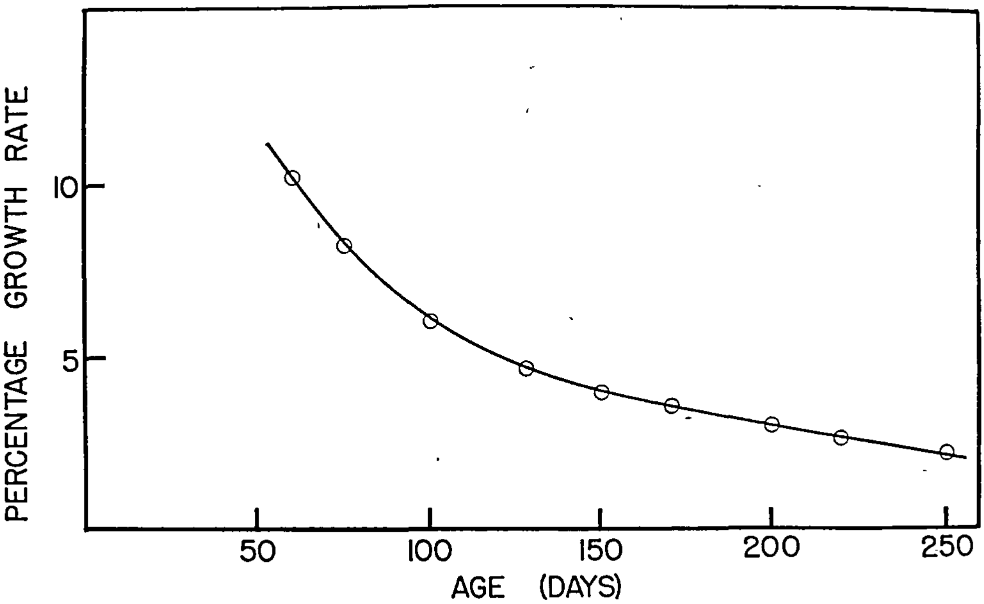


FIG. 4. Plot of percentage growth rate of the fetal thyroid against age.

resembles that obtained by Brody (1941) for the percentage growth of the thyroid in the postnatal state.

## SUMMARY

The growth of the thyroid gland, in relation to body weight, body length, and age, in the bovine fetus from 62 days to term was investigated. Fetal age was calculated from the following four parameters: body weight, crown-rump length, chest circumference, and abdominal circumference.

The simple allometry equation $y = bx^k$ was found to fit the data for the growth of the thyroid in relation to body weight, body length, and age. The relative growth constant ($k$) for thyroid weight against body weight was found to be 1.0. This indicates that thyroid weight in the fetus is nearly directly proportional to body weight within the limits of the empirical formula.

Percentage growth rates were calculated and found to decrease with increasing age.

## ACKNOWLEDGMENT

We are indebted to H. B. Knowles, Jr., for assistance in the mathematical treatment of the data presented here. We wish to acknowl-

edge our indebtedness to Lewis and McDermott, Berkeley, and to H. Moffatt Company, San Francisco, for their cooperation in furnishing the fetal material used in this study.

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# THE ACCUMULATION OF THYROXINE-LIKE AND OTHER IODINE COMPOUNDS IN THE FETAL BOVINE THYROID[1]

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THE allometric constant ($k$ in the equation $y = bx^k$) for thyroid weight as a function of body weight in the fetal calf was shown to be 1.0. This indicates that the growth of the thyroid gland in the fetal calf is almost directly proportional to body weight. Although the above formula was originally used in connection with morphological dimensions, it has been widely applied to chemical magnitudes by Needham (1942) who has termed such studies chemical heterogeny. Thus, in the allometric equation $y = bx^k$, $y$ becomes the chemical entity and $x$ the morphological magnitude (body weight, crown-rump (C.R.) length, or age in days).

In the present investigation, the accumulation of two chemical fractions, thyroxine-like and non-thyroxine iodine, was observed in the fetal calf thyroid from 53 days to term. The amounts found were correlated with body weight, C.R. length, and age of the fetus. The results obtained show that the accumulation of both iodine fractions in the fetal calf thyroid is related to the growth parameters (body weight and C.R. length) in a manner similar to that of the growth of the gland itself.

## EXPERIMENTAL

Fetal calf thyroids were collected as described in the preceding paper. A total of 96 thyroids was obtained from fetuses ranging in age from 53 days to term. Fifty-five were males and 41, females. The dams were of the following breeds: Hereford, 82; Guernsey, 2; Holstein, 9; and Jersey, 3.

The thyroids were excised from the fetuses approximately one hour after death of the mothers. Representative portions of most of the glands were hydrolyzed on a steam bath for 12 hours in 2N NaOH (20 ml. per gm. wet weight). A suitable aliquot of the hydrolysate was analyzed for thyroxine-like and non-thyroxine iodine according to the method of Taurog and Chaikoff (1946).

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[2] U. S. Public Health Fellow.

In those cases in which organic and inorganic iodine were separated, the glands were homogenized in an all-glass apparatus with cold 10 per cent trichloroacetic acid (10 ml. per gram). The precipitate obtained after centrifugation was washed twice with cold five per cent trichloroacetic acid. The supernatants, which contained all of the inorganic fraction, were combined and analyzed as previously described (Taurog and Chaikoff, 1946b). The trichloroacetic acid soluble (organic) fraction was hydrolyzed in 2N NaOH, and treated as described in the preceding paragraph.

## RESULTS

Thyroid iodine of 16 fetuses was separated into trichloroacetic acid-soluble and trichloroacetic acid-precipitable iodine. The results are recorded in table 1. The trichloroacetic acid-soluble fraction constituted, as a rule, 11 per cent or less of the total iodine. In only one case was a value above 11 per cent found. It is evident, therefore,

TABLE 1. THE ORGANIC AND INORGANIC FRACTIONS OF FETAL CALF THYROIDS

| Calculated fetal age | Thyroid weight | Iodine | | |
|---|---|---|---|---|
| | | Organic | Inorganic | Inorganic as per cent of total iodine |
| days | mg. | gamma | gamma | |
| 64 | 1 | 0.94 | 0.10 | 9.6 |
| 68 | 9 | 1.2 | 0.22 | 18.5 |
| 75 | 9 | 0.84 | 0.10 | 10.6 |
| 78 | 17 | 1.0 | 0.08 | 7.6 |
| 80 | 18 | 3.4 | 0.34 | 9.1 |
| 85 | 24 | 0.98 | 0.08 | 7.5 |
| 107 | 133 | 14.6 | 0.24 | 1.6 |
| 108 | 111 | 17.0 | 0.21 | 1.2 |
| 120 | 255 | 25.4 | 2.6 | 9.3 |
| 125 | 345 | 55.2 | 2.2 | 5.9 |
| 143 | 895 | 129 | 12.1 | 8.5 |
| 154 | $1.13 \cdot 10^3$ | 120 | 14.6 | 10.8 |
| 168 | $2.37 \cdot 10^3$ | 424 | 33.9 | 7.4 |
| 172 | $2.63 \cdot 10^3$ | 360 | 34.2 | 8.6 |
| 198 | $4.32 \cdot 10^3$ | $2.54 \cdot 10^3$ | 198 | 7.2 |
| 252 | $8.00 \cdot 10^3$ | $2.59 \cdot 10^3$ | 76.5 | 2.8 |

TABLE 2. NATURE OF THYROID IODINE OF THE ADULT BOVINE
(All females approximately 6 years old)

| Thyroid weight | Iodine Contents | | |
|---|---|---|---|
| | Organic | Inorganic | Inorganic as per cent of total iodine |
| gm. | mg. | gamma | |
| 16.2 | 25.9 | 450 | 1.7 |
| 17.0 | 14.9 | 259 | 1.7 |
| 14.0 | 15.3 | 305 | 1.5 |
| 18.0 | 34.1 | 345 | 1.0 |

TABLE 3. NATURE OF IODINE IN FETAL CALF THYROID

| Age range | Number of fetuses examined | Fetal weight | | Thyroid | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Weight | | Total iodine | | Thyroxine iodine | | Non-thyroxine iodine | |
| | | Range | Mean | Range | Mean | Range | Mean | Range | Mean | Range | Mean |
| days | | gm. | gm. | mg. | mg. | gamma | gamma | gamma | gamma | gamma | gamma |
| 53– 59 | 3 | 6.7 –12.7 | 8.3 | — | — | <0.1 – 0.40 | — | <0.1 | — | <0.1 – 0.35 | — |
| 60– 68 | 6 | 12.7 –34 | 24 | 1– 9 | 3.4 | 0.91– 1.2 | 0.95 | 0.1 – 0.36 | 0.15 | 0.58– 1.0 | 0.70 |
| 70– 80 | 6 | 34– 67 | 52 | 4– 19 | 12.5 | 0.26– 3.8 | 1.9 | 0.1 – 0.79 | 0.44 | 0.51– 2.9 | 1.34 |
| 82– 88 | 8 | 79–156 | 119 | 16– 78 | 38 | 1.8 – 7.8 | 4.1 | 0.42 – 3.5 | 1.53 | 0.56– 5.8 | 2.6 |
| 90– 98 | 7 | 96–240 | 179 | 37– 78 | 54 | 3.3 –15.5 | 6.0 | 0.5 – 4.0 | 1.4 | 2.8 –11.5 | 4.7 |
| 100–105 | 9 | 285–490 | 371 | 57–183 | 106 | 4.6 –20.0 | 9.9 | 0.95 – 5.0 | 2.7 | 3.6 –15.0 | 8.3 |
| 106–110 | 7 | 438–555 | 491 | 110–178 | 131 | 7.7 –26.6 | 16.4 | 1.7 –11.1 | 4.6 | 4.6 –18.8 | 11.8 |
| | | kg. | kg. | | | | | | | | |
| 118–125 | 5 | 0.71– 1.32 | 0.89 | 155–435 | 243 | 42.3 –63.9 | 49.7 | 9.8 –38.3 | 17.9 | 25.1 –39.6 | 31.8 |
| 126–138 | 9 | 0.14– 1.75 | 1.44 | 290–470 | 379 | 26.6 –127 | 96.6 | 15.8 –36.6 | 26.5 | 10.8 –91.2 | 70.0 |
| | | | | gm. | gm. | | | | | | |
| 140–160 | 12 | 1.80– 3.81 | 2.54 | 0.42– 1.13 | 0.75 | 135–423 | 252 | 28.0 –118 | 60.6 | 82.5 –351 | 189 |
| 163–170 | 6 | 4.34– 5.50 | 4.81 | 1.13– 2.52 | 1.87 | 315–805 | 580 | 75.4 –300 | 212 | 190–550 | 362 |
| | | | | | | mg. | mg. | mg. | mg. | mg. | mg. |
| 172–205 | 6 | 5.63– 9.60 | 8.55 | 2.43– 5.07 | 3.56 | 0.40– 2.74 | 1.95 | 0.078– 1.01 | 0.51 | 0.29– 1.88 | 1.40 |
| 215–218 | 3 | 12.5 –15.7 | 14.3 | 4.71– 6.95 | 6.02 | 3.21– 5.56 | 4.27 | 1.07 – 1.54 | 1.35 | 2.14– 4.02 | 3.00 |
| 240–255 | 6 | 19.9 –26.8 | 23.7 | 4.60– 8.00 | 5.97 | 2.67– 9.20 | 6.28 | 0.69 – 2.63 | 1.88 | 1.90– 6.58 | 4.38 |
| 260–265 | 2 | 32.5 –40.4 | — | 10.3 –11.4 | — | 4.96–17.4 | — | 1.40 – 5.32 | — | 3.56–12.1 | — |

that the major proportion of thyroid iodine in the fetus is organic. The values obtained by trichloroacetic acid separation of the adult bovine thyroid are shown in table 2.

The iodine values for thyroxine and non-thyroxine in the fetal thyroids have been arbitrarily grouped in table 3 according to the calculated ages of the fetuses. The values for the new-born calf are recorded in table 4.

TABLE 4. NATURE OF THYROID IODINE OF NEW-BORN CALVES
(All males)

| Thyroid weight | Iodine contents | | |
|---|---|---|---|
| | Total iodine | Thyroxine-like iodine | Non-thyroxine iodine |
| gm. | mg. | mg. | mg. |
| 6.95 | 6.76 | 2.08 | 4.68 |
| 7.21 | 8.48 | 2.40 | 6.08 |
| 2.63 | 3.16 | 0.93 | 2.23 |
| 4.51 | 6.42 | 1.60 | 4.82 |
| 4.00 | 4.42 | 1.04 | 3.38 |

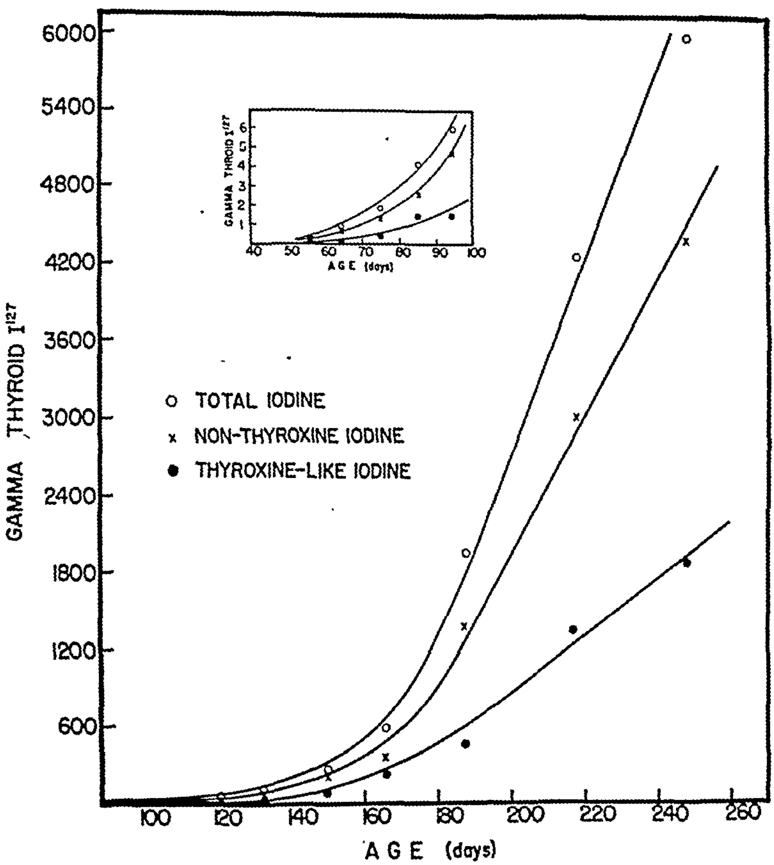


FIG. 1. Plot of fetal thyroid iodine against calculated age.

Despite considerable variation in the individual values for each age group, the means show that the iodine content of the fetal thyroid increases steadily with increasing body weight, C.R. length, and calculated age (table 3).

The average values for thyroxine-like and non-thyroxine iodine are plotted against calculated age in fig. 1. The curves suggest an ex-

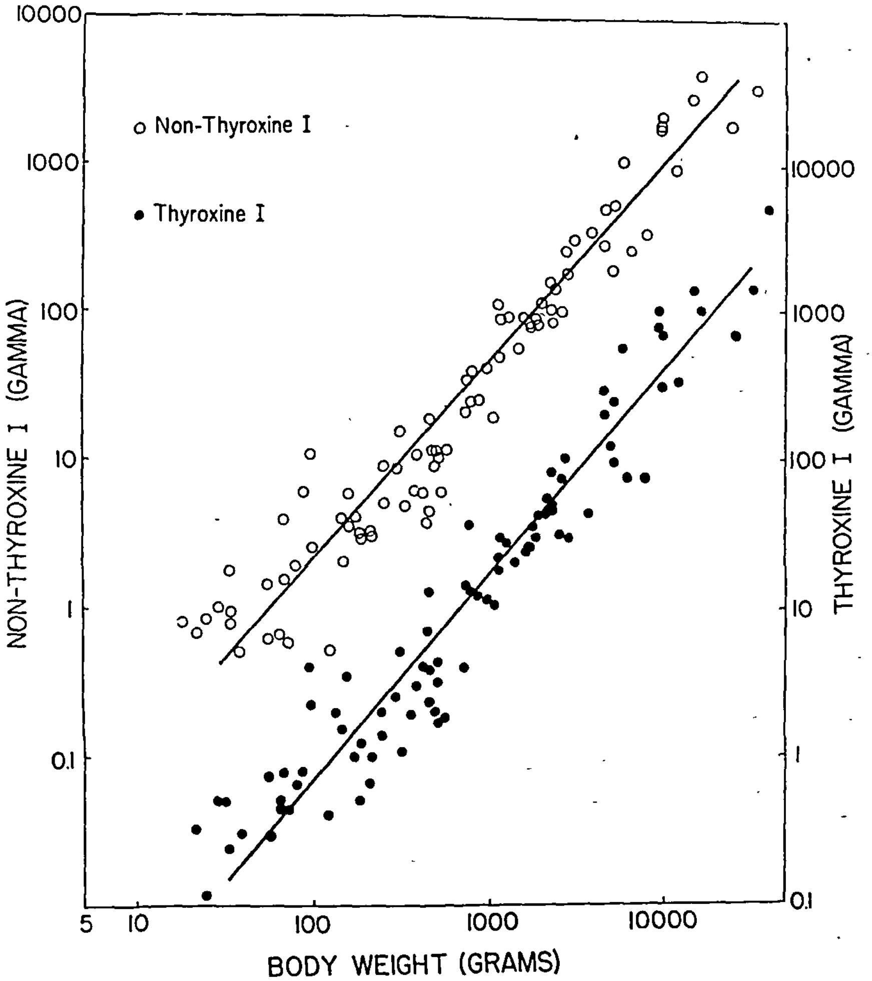


FIG. 2. Double-logarithmic plot of fetal thyroid iodine against body weight.

ponential relation between these iodine fractions and the age parameter. The chemical entities (thyroxine-like, non-thyroxine iodine) were therefore plotted against body weight, C.R. length, and age (figs. 2–4) on a double logarithmic grid. The straight-line curves obtained were subjected to analysis by application of the allometry equation of Huxley (1932), $y = bx^k$.

*Relation to Body Weight*

The equation for thyroxine-like iodine as a function of body weight (fig. 2) is

$$y_1 = (1.2 \cdot 10^{-3})x^{1.4} \quad (1)$$

where $y_1$ = thyroxine-like iodine in gamma.
$x$ = body weight in gm.
The constants $b$ $(1.2 \cdot 10^{-3})$ and $k$ (1.4) were derived as described in the preceding paper.

Likewise, the equation for non-thyroxine iodine against body weight shown in fig. 2 is

$$y_2 = (3.1 \cdot 10^{-3})x^{1.3} \quad (2)$$

where $y_2$ = non-thyroxine iodine in gamma,
$x$ = body weight in gm.

These formulae are valid for a body weight range from 50 gm. to 20 kg.

The constants expressing the "growth" of the chemical entities (1.4 for thyroxine-like iodine; 1.3 for non-thyroxine iodine) indicate that thyroxine-like and non-thyroxine iodine increase at approximately the same rate in the growing calf fetus.

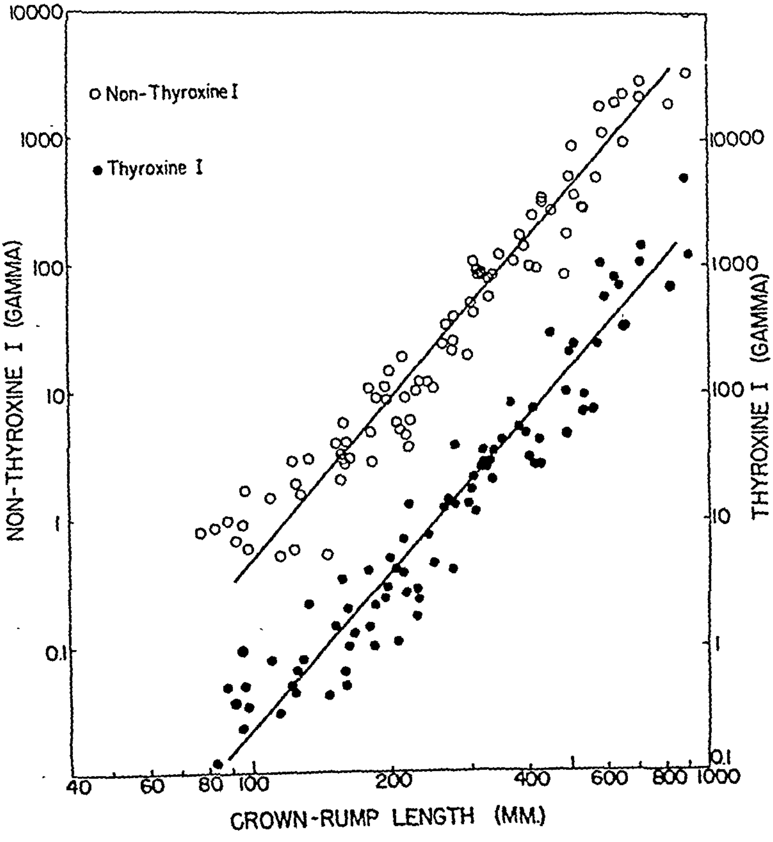


FIG. 3. Double-logarithmic plot of fetal thyroid iodine against crown-rump length.

*Grown-Rump Length Relations*

The curves for thyroxine-like and non-thyroxine iodine plotted against C.R. length are shown in fig. 3. The following equation fits the data for thyroxine-like iodine against C.R. length:

$$y_1 = (1.3 \cdot 10^{-9})x^{4.1} \tag{3}$$

in which $x$ = C.R. length in mm.
$y_1$ = thyroxine-like iodine in gamma.

The formula for non-thyroxine iodine plotted against C.R. length is

$$y_2 = (2.1 \cdot 10^{-9})x^{4.2} \tag{4}$$

The above expressions are valid for C.R. lengths extending from 100 to 800 mm.

A comparison of the two equations (where $k$ is 4.1 and 4.2, respectively) indicates that thyroxine-like and non-thyroxine iodine increase at about the same proportion in relation to body length.

*Age Relations*

A plot of thyroxine-like and non-thyroxine iodine against age yields curves having the following formulae

$$y_1 = (5.7 \cdot 10^{-10})x^{5.4} \tag{5}$$

$$y_2 = (1.8 \cdot 10^{-10})x^{5.3} \tag{6}$$

where $y_1$ and $y_2$ represent thyroxine-like and non-thyroxine iodine, respectively, in gamma, and $x$, the age in days. The validity of the formulae for thyroxine-like and non-thyroxine iodine against age extend from 60 days to term.

The amounts of these two iodine fractions in the fetal calf thyroid increase at approximately the same proportion with increasing fetal age. The values for $k_1$ and $k_2$ were 5.4 and 5.3, respectively.

## DISCUSSION

As the fetus grows, total and thyroxine-like iodine increase steadily in the gland, and the rate at which each of these iodine fractions accumulates is shown to bear an exponential relation to body weight and body length as well as to calculated age. The accumulation of these two iodine fractions in the fetal gland is not only the result of thyroid growth (which, as shown in the previous paper, is also exponential) but, in addition, appears to be the result of an actual increase in the capacity of thyroid tissue to store iodine with increasing age. This increasing iodine-storage capacity of thyroid tissue, with age, is shown in fig. 5 which shows the *concentration* of iodine fractions in the gland (mg. iodine per 100 gm. of fresh tissue) against fetal age.

It was previously demonstrated, in 11 vertebrates, including the rat, examined under a variety of experimental conditions, that thyroxine iodine, when expressed as a percentage of total iodine of the gland, remains fairly constant (Wolff *et al.*, 1947; Taurog *et al.*, 1946c, 1946d). The values for this percentage ranged from 25 to 32. It therefore became of interest to determine whether a similar relation existed in the fetal calf thyroid.

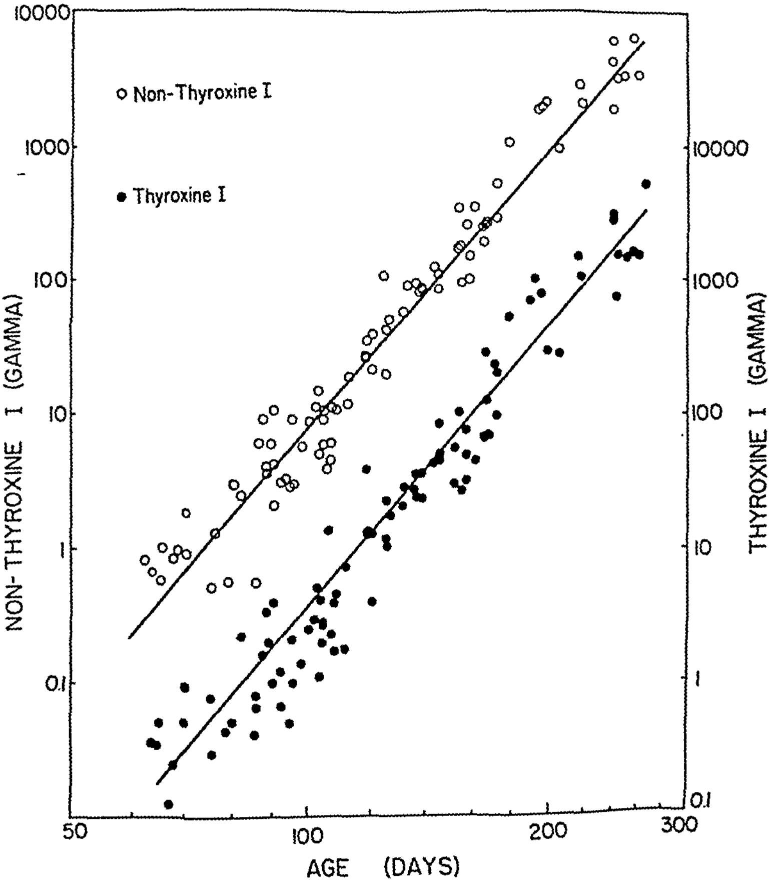


FIG. 4. Double-logarithmic plot of fetal thyroid iodine against calculated age.

The parallelism between the two curves shown in each figure (2, 3, 4, and 5) suggests that in the fetal calf, also, thyroxine iodine comprises a constant proportion of total thyroid iodine. An examination of the ratios for individual thyroids of fetuses obtained during the last third of the period of gestation revealed a constancy in the proportion of thyroxine-like iodine to total iodine. Eighteen specimens that were 170 days or older were studied, and with the exception of a single ratio, all values fell within the range of 24 to 36 per cent. This constancy in the proportion of thyroxine iodine to total iodine was

first observed with frequency, in fetuses older than 120 days. But in younger fetuses, the proportion varied considerably, most of the values falling below 20 per cent. The deviations from 24–36 per cent were equally divided between the sexes and did not appear to be related to the absolute amounts of iodine contained in these glands.

Rankin (1941) investigated the appearance of iodine in the thyroid of the fetal pig, which has a gestation period of 114 days. He first found inorganic iodine in the gland between 46 and 50 days of age, and organic iodine at 52 days of age. As shown here, thyroxine-

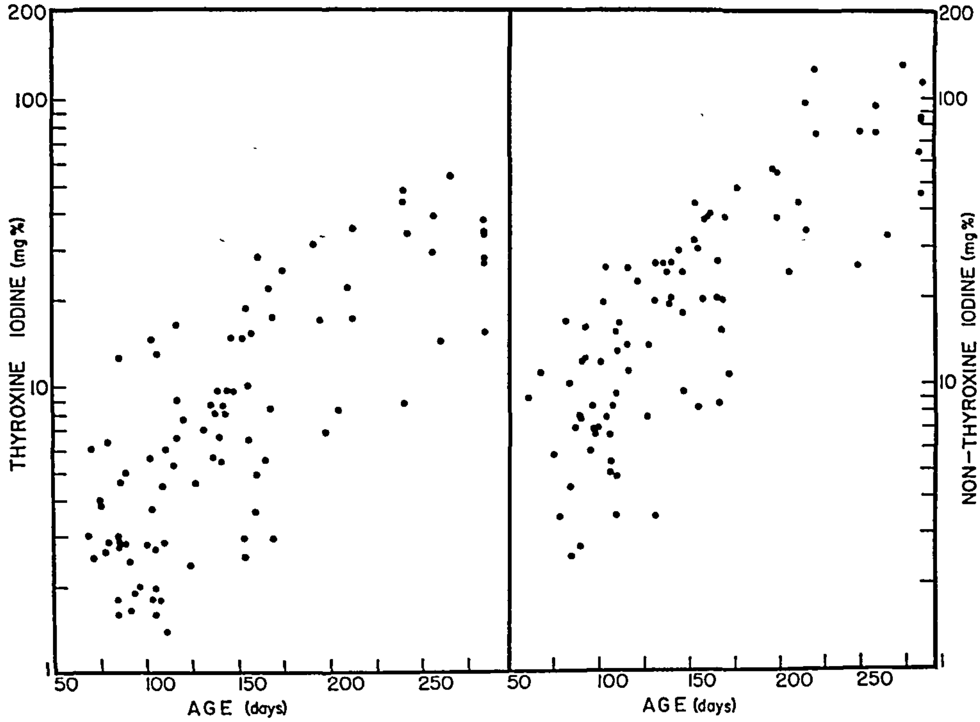


Fig. 5. Semi-logarithmic plot of the concentration of fetal thyroid iodine against calculated age.

like iodine appears at an earlier stage of gestation in the fetal calf, being first detected at 60 days of age (gestation period, 278–285 days).

Although the present investigation clearly demonstrates that thyroxine-like and non-thyroxine iodine increase proportionately with increasing fetal size and age, it provides no information on the nature of the iodine taken up by the fetal thyroid gland. It is unlikely that the iodine was taken up in the form of thyroxine or diiodotyrosine (Leblond, 1942). The iodine probably enters the fetal gland in the inorganic form as it does in the adult. Gorbman and Evans (1943) have demonstrated, in the fetal rat, that functional activity of the thyroid gland (as judged by its capacity to concentrate radioactive inorganic iodine) begins at 18–19 days of gestation. Preliminary experiments with surviving slices of fetal calf thyroid have shown that

this tissue also has the capacity to concentrate inorganic iodine and that this capacity appears at a considerably earlier stage in the developing fetal calf than in the rat fetus.

SUMMARY

The amount of inorganic, thyroxine-like, and non-thyroxine iodine were measured in the bovine fetal thyroid from 53 days to term.

Measurable amounts of iodine were first detected in the fetal thyroid at 60 days of age.

The percentages of total iodine present as inorganic were similar to those observed in the adult thyroid gland.

The accumulation of thyroxine-like and non-thyroxine iodine in the fetal thyroid is related to each of the following growth parameters: body weight, crown-rump length, and calculated age. A straight-line relationship was found to exist when these iodine fractions were plotted against each of the parameters on a double-logarithmic grid.

The amounts of iodine found in the bovine fetal thyroid with increasing age were greater than could be accounted for by mere increase in thyroid mass. A progressive increase in the iodine concentrating capacity of fetal thyroid tissue occurred with age.

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# RELATIONSHIPS OF DIET TO THE DURATION OF SURVIVAL, BODY WEIGHT AND COMPOSITION OF HYPOPHYSECTOMIZED RATS[1]

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SURGICAL removal of the pituitary gland has been known for many years to be compatible with life but investigators using such pituitary-less mammals have learned with dismay that this is a qualified truth. Animals lacking this gland are at best frail creatures with no ability to adapt themselves either to physiological stresses nor to environmental vicissitudes. Their survival is possible only under controlled laboratory conditions.

In this laboratory, large numbers of hypophysectomized rats have been used for a variety of experimental procedures in which no particular attention has been paid to their diet other than to offer them laboratory chow *ad libitum*. Under these circumstances, it has been repeatedly observed that a variable mortality had to be reckoned with in both treated and control groups in experiments lasting much beyond 10 days after operation. For example, in one untreated group of 13 hypophysectomized young male rats, 10 survived until the 8th post-operative day, and 6 until the 22nd day; in another untreated group of 18 young male hypophysectomized rats, only 8 survived until the 30th day. In a third untreated group of 12 male rats, 7 died before the 15th post-operative day and an additional 4 within the next 28 days (Greep and Deane, unpublished data). In striking contrast, preliminary observations in this laboratory indicated that when hypophysectomized rats were maintained on purified rations adequate in all known nutritional essentials, a high percentage survived for several weeks, and the general appearance of the animals remained quite good. Since many experiments in which hypophysectomized rats are used would benefit by long periods of survival, any experimental regimen on which a high percentage of the operated rats could be maintained consistently for several weeks or months would be of distinct value.

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This preliminary investigation was designed to test the extent of the differences in the percentage survival and in the duration of survival when hypophysectomized male rats were maintained on commercial laboratory chow and on purified diets. In addition, tests were made to determine whether some of the more obvious physical and chemical differences between laboratory chow pellets and granular purified rations were responsible for their different effects on the well being of the hypophysectomized rat.

## EXPERIMENTAL PROCEDURE

In the first experiment, 46 male albino rats of the Sprague-Dawley strain, which were 28 days of age, were hypophysectomized by the parapharyngeal approach during one day. Then, the animals were immediately distributed into 5 groups, in such a way that the range and average body weights of the rats in any group were almost identical to the respective values in any other group. Each rat was placed in a small individual cage with wire bottom and supplied with water *ad libitum* by a freely flowing watering bottle. One group was fed a commercial laboratory chow ground sufficiently fine to pass readily through a 20-mesh sieve, thereby approximating the particle size of the purified rations. This was deemed necessary as a test of whether the hard pellets of laboratory chow were sufficiently difficult for lethargic, hypophysectomized rats to nibble and masticate as to reduce their food consumption to a sub-optimal amount. Another group was offered the same chow in its original pellet form.

The other 3 groups were given purified rations, the basic components of which are recorded in Table 1. These diets are adequate for the normal rat in all known nutritional essentials. One of these groups received a high carbohydrate ration (100) which had an energy value of about 4.1 calories

TABLE 1. COMPOSITION OF THE PURIFIED RATIONS FED TO HYPOPHYSECTOMIZED RATS

| Ration components | Ration | | |
|---|---|---|---|
| | 100 | 100+20% cellu flour | 150 |
| | gm. | gm. | gm. |
| Sucrose | 67 | 67 | 31 |
| Casein[1] | 24 | 24 | 24 |
| Salts[2] | 4 | 4 | 4 |
| Corn oil[3] | 5 | 5 | 5 |
| Lard | — | — | 16 |
| Cellu flour[4] | — | 20 | — |
| Whole liver substance[5] | 2 | 2 | 2 |
| 1:20 liver concentrate powder[5] | 2 | 2 | 2 |

[1] Casein—"De-vitaminized" casein (Sheffield Farms, New York, N. Y.) to each 24 gm. of which has been added 350 µg. thiamine hydrochloride, 350 µg. riboflavin, 350 µg. pyrixodine hydrochloride, 2.5 mg. nicotinic acid, 2.0 mg. calcium pantothenate, 100 mg. choline chloride, 100 mg. inositol and 30 mg. para-aminobenzoic acid.

[2] Corn oil—Mazola to 5 gm. of which has been added 1.1 mg. beta-carotene, 300 I.U. irradiated ergosterol, 0.6 mg. 2-methyl-1, 4-naphthoquinone and 5.0 mg. alpha-tocopherol.

[3] J. Dental Research 26, 47 (1947).

[4] Chicago Dietetic Supply House, Chicago, Ill.

[5] The Wilson Laboratories, Chicago, Ill.

per gram. A second group was given the same ration to which had been added 20 per cent Cellu flour as a source of crude, undigestible fiber (100+20 per cent Cellu flour); the energy value of this ration was about 3.5 calories per gram, which closely approximated the energy value of the laboratory chow. It has been found that the addition of this amount of crude fiber to purified rations does not appreciably affect its nutritional properties when the diet is offered *ad libitum.*

As a reverse evaluation of any possible energy relationship, the energy value of the purified ration per gram was increased by the isocaloric replacement of lard for part of the sucrose. Thus a third group was offered ration 150 in which approximately one-half of the carbohydrate had been replaced by lard (16 grams of lard substituted for 36 of each 67 grams of sucrose). This ration had a caloric value of about 5.1 calories per gram. All diets were made available to the rats *ad libitum.*

At the end of 88 days, the 16 surviving animals were killed with chloroform and the weights of the adrenal, thymus, testes, seminal vesicles and testicular fat bodies determined on a Roller-Smith torsion balance. Ratios of weights of these organs to body weight were calculated as indices of the activity of the related endocrine systems. The sella turcica of each animal was examined for evidence of any remnants of the hypophysis. Radiographs were made of the tails of 1) the survivors in experiment 1; 2) 10 rats with body weights comparable to those of the survivors at the time of hypophysectomy; and 3) 10 rats with body weights corresponding to the autopsy weights of the hypophysectomized rats. From the latter group, the organs mentioned above were weighed.

The average length of the last 22 caudal vertebrae, the 12th caudal vertebra and the 12th caudal intervertebral space were measured for the survivors and the controls as tests of whether tail growth had occurred.

In the second experiment, 34 male albino rats of the same strain and age were hypophysectomized on the same day and immediately divided into three similar groups which were housed as in the first experiment and fed ground chow, the high carbohydrate purified diet (100) and the high fat purified diet (150) *ad libitum,* respectively. In this case, the food consumption per rat per day was determined for the first 70 days of the experiment. On the 112th day, 4[2] of the 14 survivors were saved for longevity studies and for histologic purposes; the remaining 10 were sacrificed and the same organs weighed as in the first experiment. All these tissues except those needed for histological study were placed with the rest of the carcass after weighing; then analyses of the carcasses minus the contents of the gastrointestinal tract, were made for water, fat, ash and protein by the methods described by Deuel, Hallman, Movitt, Mattson and Wu (1944). The tails of these rats were radiographed and the same measurements made as in the initial experiment.

The testes and adrenals of representative rats from the two surviving groups of the second experiment were fixed in 10 per cent neutral formalin. Paraffin sections of the testes were stained in hematoxylin and eosin. Frozen sections of one adrenal from each animal were stained with 1) Sudan IV, 2)

[2] The four rats which were not sacrificed at 112 days were continued on their original experimental regimens. One died on the 176th post-operative day, a second was sacrificed in a weakened state on the 240th day and 2 are still alive on the 260th day.

Sudan IV and Harris' hematoxylin and 3) by the Schiff plasmal method. An additional two sections of each adrenal were mounted unstained in glycerin jelly, one was untreated, the other extracted for one-half hour in acetone at room temperature. These latter were studied under the polarizing microscope for acetone-soluble droplets showing birefringence and under the flourescent microscope for the presence of autofluorescing material.

All animals in both experiments were weighed every other day throughout the experimental periods. Each morning, the numbers of animals which had died in the previous 24 hours were recorded. At frequent intervals, all animals were palpated for gross evidence of increase in testicular size. In all, 4 rats were discarded from further consideration when slight testicular enlargement was noted. Any animals which died in the first 48 hours after the operation were considered to have died because of trauma incurred in the operation and not because of hypophysectomy *per se*. Of the 80 rats in these experiments, 5 died within the first 48 hours and were disregarded in all evaluations of the data.

## RESULTS

The general appearance of the hypophysectomized rats maintained on the purified rations was exceptionally good throughout the duration of the experimental periods. The hair coat remained fine and smooth in texture. The external appearance of the hypophysectomized rats maintained on the high fat ration (150) was especially sleek and well-groomed. No evidence of cyanosis at the extremities was observed except during short periods previous to death in those which

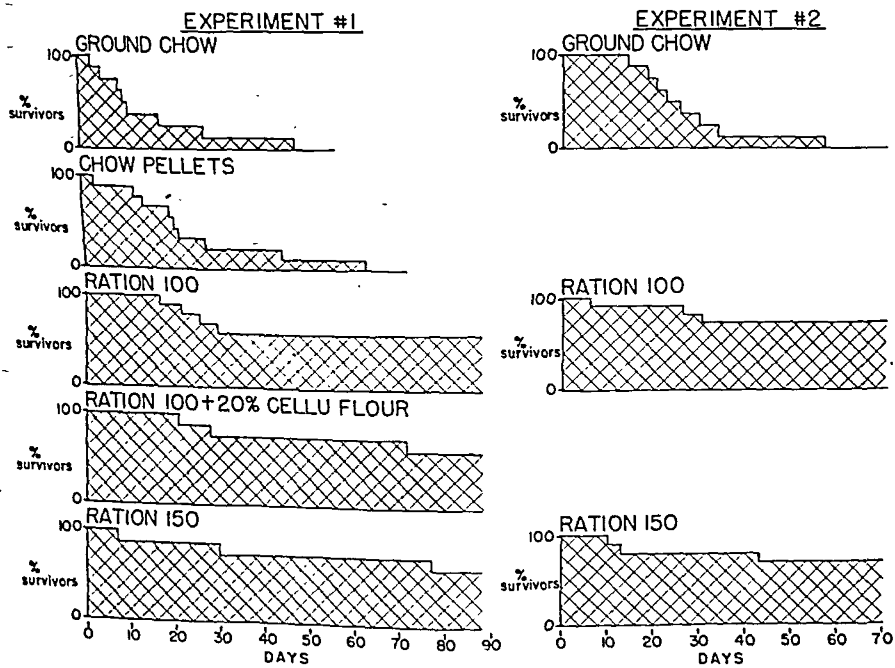


FIG. 1. Graphical representation of the percentage survival of hypophysectomized rats maintained on the various rations in experiments 1 and 2.

TABLE 2. NUMBER OF SURVIVORS AND THEIR CHANGES IN BODY WEIGHT AS OF THE 7TH, 35TH, AND 70TH POST-OPERATIVE DAYS AND AT THE END OF THE EXPERIMENTS

| Ration | Initial no. of rats* | Avg. wt. | 7th post-operative day | | | 35th post-operative day | | | 70th post-operative day | | | 88th post-operative day | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | No. survivors | Avg. wt. increase | Range of wt. change | No. survivors | Avg. wt. increase | Range of wt. change | No. survivors | Avg. wt. increase | Range of wt. change | No. survivors | Avg. wt. increase | Range of wt. change |
| | | gm. | | gm. | gm. | | gm. | gm. | | gm. | gm. | | gm. | gm. |
| *Experiment 1* | | | | | | | | | | | | | | |
| Ground chow | 8 | 57 | 6 | 6 | −6 to 16 | 1 | 15 | — | 0 | — | — | 0 | — | — |
| Chow pellets | 9 | 59 | 8 | 15 | 9 to 19 | 2 | 15 | 14 to 15 | 0 | — | — | 0 | — | — |
| 100 | 10 | 64 | 10 | 6 | −7 to 16 | 6 | 13 | −2 to 29 | 6 | 38 | 7 to 55 | 6 | 43 | 7 to 63 |
| 100+20% Cellu flour | 8 | 64 | 8 | 8 | −7 to 14 | 6 | 20 | −9 to 32 | 6 | 23 | −1 to 33 | 5 | 28 | 4 to 41 |
| 150 | 8 | 61 | 7 | 12 | 3 to 20 | 6 | 24 | 14 to 37 | 6 | 37 | 20 to 58 | 5 | 49 | 38 to 69 |
| *Experiment 2* | | | | | | | | | | | | 112th post-operative day | | |
| Ground chow | 8 | 50 | 8 | 9 | −1 to 28 | 1 | 13 | — | 0 | — | — | 0 | — | — |
| 100 | 11 | 50 | 10 | 11 | −3 to 19 | 8 | 27 | 7 to 50 | 8 | 39 | 13 to 76 | 7 | 55 | 40 to 93 |
| 150 | 10 | 48 | 10 | 9 | −7 to 25 | 8 | 24 | 6 to 41 | 7 | 39 | 7 to 61 | 7 | 52 | 24 to 76 |

* Rats which were found to have partial testicular maintenance during the experimental period, a remnant of the hypophysis at post-mortem, or which died from operative trauma were not included in these numbers.

did not survive the experimental periods. In contrast, within a few hours after hypophysectomy, the rats fed either ground chow or chow in pellet form began to appear unkempt and cyanotic and often remained thus until death. The animals fed chow as pellets or in ground form were also extremely lethargic in contrast to the relative activity of those rats maintained on the purified ration. No difference was noted in behavior or general appearance between the rats on the two forms of chow.

The effect of the various diets upon the duration of survival of hypophysectomized rats is presented in Figure 1 and in Table 2. In experiment 1, the duration of survival and the percentage of survivors among the 3 groups of rats fed the purified rations (100, 100+20 per cent Cellu flour, and 150) were significantly higher than among the groups of rats fed chow pellets or ground chow. No significant differences were observed in the survival times and percentage of survivors among the 3 groups of rats fed the different purified rations, nor were any significant differences observed between the groups fed the two physically different forms of chow. In experiment 2, a significantly longer duration of survival and a significantly higher percentage of survivors was observed among the hypophysectomized rats fed the 2 purified rations than among those rats fed ground chow. Again, no differences were observed in survival between the groups fed the two purified rations.

During the first few days after hypophysectomy, numerous rats in each of the dietary groups lost appreciable amounts of body weight while others in each group maintained their original weight and some began to gain immediately. On the 7th day of the first experiment, the average weight increase for each group varied from 6 to 15 grams. At this time, the rats fed chow in pellet form and those fed ration 150 had increased in body weight on the average slightly more than those animals in the other 3 groups. On the 35th day, the 6 surviving animals fed ration 150 had gained on the average slightly more than the 6 fed 100+20 per cent Cellu flour and these in turn had gained slightly more than the surviving 2 rats in the ground chow group, the 1 survivor in the chow pellet group and the 6 rats in the ration 100 group. By the 70th day and 88th days, there were no survivors in the groups receiving chow but those hypophysectomized rats fed rations 100 and 150 were equal in average weight gain and had gained appreciably more than those rats fed the ration with added undigestible crude fiber. The average increase in body weight for the rats fed rations 100 and 150 for 88 days was slightly in excess of two-thirds of the body weights at the time of hypophysectomy.

In experiment 2, the average weights of the rats in the 3 groups on the 7th day post-operatively were 9 to 11 grams more than at the time of the operation. By the 35th day, the average weight increases in rats fed the purified rations were double that observed in the one

survivor in the group fed ground chow. On the 70th and 112th postoperative days, the average weight increase of the surviving hypophysectomized rats fed ration 100 was almost identical with that of those rats fed ration 150 throughout the experimental period. It is interesting to note that the average weight of the rats fed rations 100 and 150 for 112 days was slightly more than double the average initial body weights.

Food consumption expressed as the average number of calories consumed per rat per day was almost identical for the 2 groups fed the purified rations and slightly exceeded that of the rats fed ground chow.

The average ratios of organs to body weight, expressed as mg. per 100 gm. of body weight, are presented in table 3 for the survivors of experiment 1 and for the 10 animals in experiment 2 which were

TABLE 3. THE AVERAGE PROPORTION OF TISSUE WEIGHTS TO BODY WEIGHTS EXPRESSED AS MG. PER 100 GM. OF BODY WEIGHT

| Exp. no. | Duration days | Ration | No. of rats | Body weight Initial | Body weight Final | Adrenal prop. | Thymus prop. | Testes prop. | Seminal vesicle prop. | Testicular fat prop. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | gm. | gm. | | | | | |
| 1 | 88 | 100 | 6 | 63 | 104 | 8.4 | 229 | 120 | 7.0 | 680 |
| | | 100+20% Cellu flour | 5 | 63 | 89 | 8.2 | 204 | 137 | 8.5 | 590 |
| | | 150 | 5 | 63 | 108 | 8.8 | 234 | 121 | 7.9 | 1070 |
| 2 | 112 | 100 | 5 | 50 | 107 | 10.5 | 190 | 67 | 6.0 | 815 |
| | | 150 | 5 | 52 | 104 | 10.1 | 160 | 100 | 6.9 | 871 |
| Control | 0 | Chow | 10 | — | 107 | 21.8 | 455 | 962 | 34.9 | 355 |

sacrificed for carcass analysis on the 112th day. These data do not include any animals on chow for the reason that none survived the experimental period. The very low ratios of the testes and of the seminal vesicles to body weight reflect the withdrawal of gonadotropic hormones due to the complete removal of the hypophysis and indicate further that this atrophic process is not influenced by the nutritional state of the animal which has permitted his survival for this length of time. The average percentile weight of the adrenals is also far below the range for intact animals of this weight but is not quite as low as that observed previously by Deane and Greep (1946) in hypophysectomized rats surviving for 56 and 136 days on chow. The average ratio of the weight of the thymus to body weight had not been reduced below the normal range for animals of equal weight.

In table 4 are recorded measurements taken from radiograms of the tails of 2 different groups of 10 intact control animals and of animals surviving on the purified diets for 88 and 112 days. The average length of the last 22 tail vertebrae in the hypophysectomized rats increased appreciably during the experimental period (Fig. 5 & 6). This increase in length is almost the same as that which occurred in normal rats during the same weight increase achieved during a shorter growth period and is apparently a result of the increase

TABLE 4. MEASUREMENTS ON THE AVERAGE LENGTH OF THE TAILS OF HYPOPHYSECTOMIZED RATS AND THEIR RESPECTIVE CONTROLS

| Experiment | Ration | No. of rats | Body weight Initial | Body weight Final | Avg. length of the last 22 tail vertebrae | Length: 12th caudal vertebra | Length: 12th caudal intervertebral space |
|---|---|---|---|---|---|---|---|
| | | | gm. | gm. | mm. | mm. | mm. |
| 1 | 100 | 6 | 63 | 104 | 106 | 5.5 | .5 |
| | 100+20% Cellu flour | 5 | 63 | 89 | 99 | 5.2 | .4 |
| | 150 | 5 | 63 | 108 | 104 | 5.3 | .5 |
| 2 | 100 | 5 | 50 | 107 | 113 | 5.9 | .4 |
| | 150 | 5 | 52 | 104 | 106 | 5.7 | .3 |
| Control | Chow | 10 | — | 64 | 78 | 3.5 | .8 |
| Control | Chow | 10 | — | 107 | 106 | 5.3 | .6 |

in length of the individual ossified vertebra. The distance between the adjacent vertebrae appears to be slightly longer in normal than in hypophysectomized rats of the same weight.

In table 5 are presented the carcass analyses for water, protein, fat, ash and carbohydrate for 2 groups of 5 rats, each of which were maintained for 112 days on ration 100 and 150, respectively. In addition, corresponding analyses are presented for these components in the carcasses of normal rats which had been maintained for 126 days on purified diets of comparable fat content by Scheer, Straub, Fields, Meserve, Hendrick and Deuel (1947). Values for normal rats fed a stock ration *ad libitum* are also listed from data of the latter investigators. The average analyses of the carcasses of the 2 groups of hypophysectomized rats do not differ appreciably from each other

TABLE 5. THE BODY COMPOSITION OF RATS FED PURIFIED RATIONS

| Ration no. | Duration | Fat content of diet | No. rats | Average body weight | Water | Protein* | Fat | Ash | Carbohydrate |
|---|---|---|---|---|---|---|---|---|---|
| | days | % | | gm. | % | % | % | % | % |
| | | | | *Hypophysectomized Rats* | | | | | |
| 100 | 112 | 5 | 5 | 105 | 57.5 | 15.2 | 19.4 | 3.9 | 4.0 |
| 150 | 112 | 25 | 5 | 103 | 57.7 | 14.3 | 20.4 | 3.9 | 3.7 |
| | | | | *Intact Rats*** | | | | | |
| 61 | 126 | 5 | 7 | 311 | 57.0 | 18.7 | 19.4 | 3.3 | 1.6 |
| 63 | 126 | 20 | 8 | 377 | 52.2 | 17.9 | 24.7 | 3.2 | 2.0 |
| Stock | 126 | 14 | 7 | 310 | 58.8 | 18.2 | 9.9 | 3.1 | 10.0 |

* We wish to acknowledge our gratitude to the Department of Nutrition of the Harvard School of Public Health for making the protein analyses of the dried, defatted carcasses of the hypophysectomized rats.

** These analyses quoted from Scheer *et al.*, J. Nutrition 34, 581 (1947).

except for the fat and protein components. The fat content of the rats fed high fat ration 150 was 20.4 per cent, whereas the fat content of the rats fed low fat ration 100 was 19.4 per cent. The average protein content of the rats fed rations 100 and 150 were 15.2 and 14.3 per cent, respectively. The carcass analyses of the hypophysectomized rats were very similar to comparable analyses of intact rats fed a low fat purified ration, except that the protein content for the 2 groups of hypophysectomized rats was appreciably lower than the 18.7 per cent value for intact rats. The carbohydrate content of the hypophysectomized rats was slightly higher than for the intact rats. Intact rats which were fed the high fat diet had a considerably higher fat content and lower water content than either group of hypophysectomized rats or than the intact rats fed the low fat purified diet. On the other hand, intact rats which were fed a stock diet composed of natural foodstuffs with an overall fat content of 14 per cent had an average carcass fat analysis of only 9.9 per cent. This low fat content of normal rats fed a stock ration indicates that the small amount of depot fat observed in hypophysectomized rats maintained in this laboratory on chow is probably a characteristic of the ration and not a result of hypophysectomy. Likewise the high fat content of the hypophysectomized rats and of the intact rats fed purified rations was apparently a result of the composition of the ration and not of the particular endocrine status of the rats *per se*.

## HISTOLOGY

The hypophysectomized rats which were given the purified rations over extended post-operative periods had the appearance of exceptionally fat animals (Fig. 2); the heavy deposits of subcutaneous fat made them appear to be padded. The skin was pinkish and glistening and could be incised with the ease of parchment. When these animals were brought to necropsy, it was revealed by gross inspection that their carcasses were indeed laden with massive deposits of both white and brown fat (Fig. 3). The testicular fat bodies were enormous. They extended the full length of the abdominal cavity and displayed many long finger-like projections which ramified amongst the loops of the intestines. Masses of brown fat were found in the interscapular region and along the posterior wall of the thoracic and abdominal cavities. Here it was in such abundance as to obscure the great vessels (Fig. 4) and the adrenal glands. The amount of brown fat in the hypophysectomized rats fed purified rations was appreciably more than in hypophysectomized rats fed chow but was approximately equal to the amount observed to be present in intact rats fed these purified rations for similar periods.

The adrenal glands from these same animals show a severe reduction of the width of the cortex (Fig. 7 & 8). The zona fasciculata was so atrophic as to make its recognition doubtful. The cells in the

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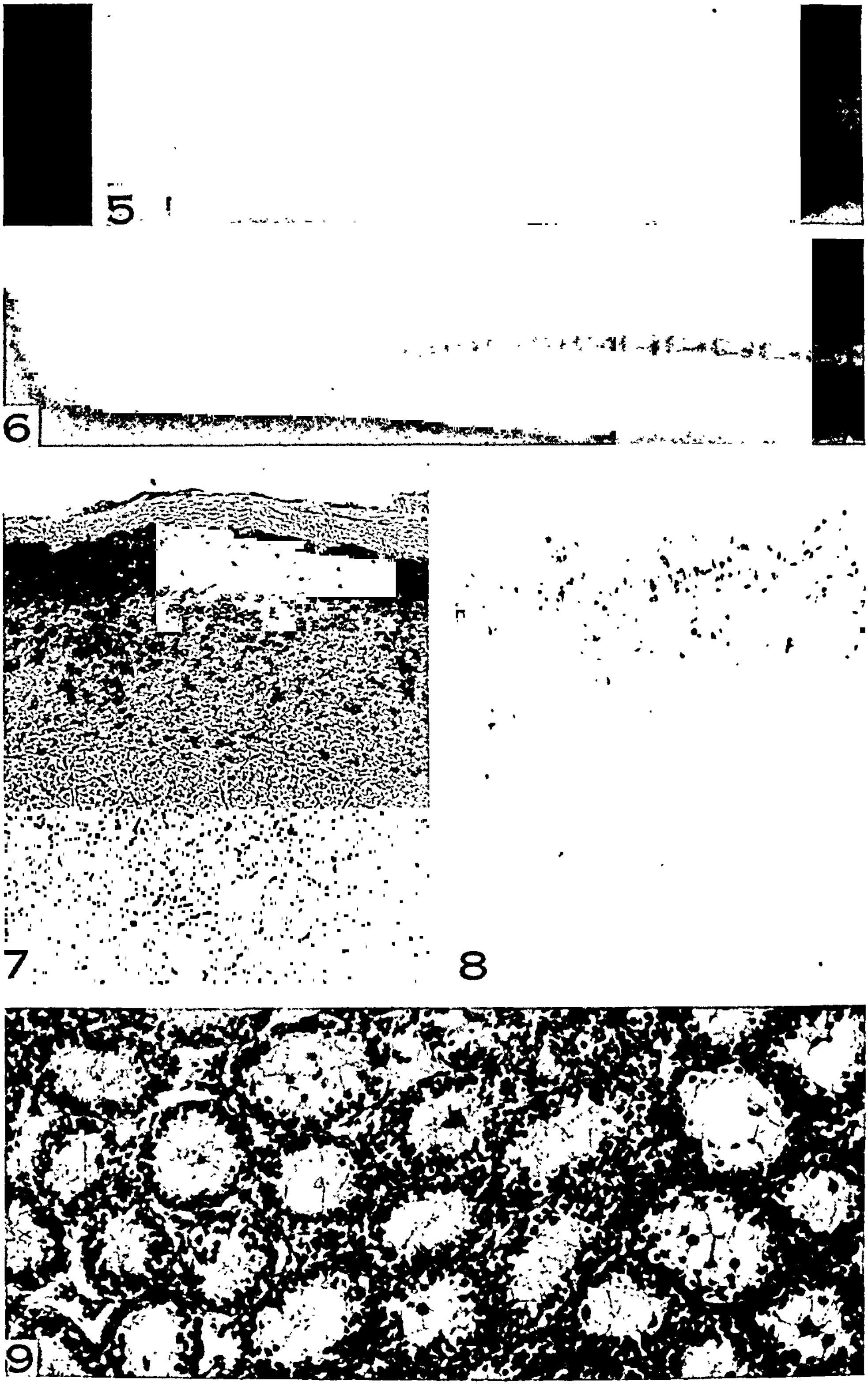
FIG. 2. A demonstration of the increase of body size of a typical hypophysectomized rat from experiment 2. The rat on the left has been maintained on ration 150 for 140 days after hypophysectomy and weighs 133 grams. The rat on the right is a normal one from the stock colony and weighs 78 grams which is the weight at which the rat on the left was hypophysectomized.

FIG. 3. Photograph of the abdominal cavity of a typical rat in experiment 2 which had been fed ration 150 for 112 days after hypophysectomy. Note the atrophic testes and the plentiful supplies of depot fat.

FIG. 4. Photograph of the thoracic cavity of the rat in figure 3 with lungs removed to show the extensive brown fat body along the dorsal aorta.



5
6
7
8
9

area between the zona glomerulosa and the medulla were shrunken, had pycnotic nuclei and were devoid of sudanophilic droplets. This area did not respond to the Schiff plasmal reagent nor did it contain any autofluorescing or doubly refractile materials.

The zona glomerulosa on the other hand was considerably broadened, its cells appeared histologically normal, and were laden with sudanophilic droplets that were also Schiff positive (Fig. 7). This zone was also strikingly autofluorescent and contained an abundance of birefrigent material that was evenly distributed as coarse and fine particles (Fig. 8). These observations are in agreement with the description of adrenal cortical changes following hypophysectomy as reported previously by Deane and Greep (1946).

The testes of 3 rats given diet 100 and 3 given diet 150 for 112 days following removal of the pituitary were examined microscopically. In all instances the seminiferous tubules and the Leydig cells were atrophic (Fig. 9). The degree of regression was sufficiently extensive to eliminate any doubt concerning the completeness of removal of the anterior lobe of the hypophysis.

## DISCUSSION

Complete surgical removal of the hypophysis is generally believed to result in cessation of growth as manifest by no further increases in body weight and skeletal dimensions. That these growth processes can be reinstituted by the growth hormone prepared from pituitary glands has been conclusively demonstrated. A point arises, however, with respect to tail growth, since Freud, Levie and Kroon (1939) concluded from a series of assay experiments, that (1) tail growth in hypophysectomized rats was more uniformly proportional to the potency of growth hormone preparations than the accompanying increases in body weight and (2) unless the hormone were injected soon after surgical removal of the pituitary, no tail growth was

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FIG. 5. Radiograph of the tail of a normal rat from the stock colony. The body weight of this rat was 63 grams at the time of this radiograph.

FIG. 6. Radiograph of the tail of a rat from experiment 1 which had been hypophysectomized at a weight of 62 grams and then maintained for 88 days on ration 100+20 per cent Cellu flour at which time it weighed 94 grams. Note the greater tail length and the increase in length and calcification of the individual vertebrae in comparison with the tail of the control animal in figure 5.

FIG. 7. Photomicrograph of a frozen section of an adrenal from a typical animal in experiment 2 sacrificed 112 days after hypophysectomy. Stained with Sudan IV. Note heavy staining of sudanophilic lipids in the zona glomerulosa of the cortex and the almost complete absence of sudanophilic lipids in the zona fasciculata. The cortex as a whole is greatly reduced in width. ×60.

FIG. 8. Photomicrograph of a frozen section of an adrenal from a typical hypophysectomized rat in experiment 2 taken with a polarizing microscope. The birefringent particles are almost entirely confined to the broadened zona glomerulosa. ×60.

FIG. 9. Photomicrograph of a section of testis from a typical hypophysectomized rat in experiment 2. The germinal cells have largely disappeared and the investiture of the tubules is composed mainly of Sertoli cells. Hematoxylin and eosin. ×60.

possible since, according to their findings, cessation of tail growth following hypophysectomy was accompanied by irreversible closure of the epiphyses of a large number of the caudal vertebrae. On the basis of our data, hypophysectomized rats maintained for relatively long periods on purified rations do not show closure of the epiphyses in the caudal vertebrae even when no growth hormone preparation was administered.

During the first 5 weeks of our experiments, the average increases in body weight of all groups of hypophysectomized rats were remarkably similar despite the great differences in the percentage survival within groups. These average increases in body weight fail to reveal an interesting difference which actually existed between the individual growth patterns of rats fed purified diets and those fed laboratory chow. In each of the 8 groups in these experiments, the weight changes of individual rats in the critical period after hypophysectomy could be divided into 3 merging categories: (1) slow but gradual and prolonged weight increases, (2) weight maintenance without appreciable fluctuation and (3) gradual weight loss. Some rats receiving ground chow or chow pellets began to lose weight after hypophysectomy and died in relatively short periods; others maintained their weight for a short interval, followed by weight loss and death. In direct contrast, rats which received either of the purified rations and began to lose weight post-operatively, survived for relatively long periods. Several recovered to the point of weight maintenance and eventually made progressive weight gains. Moreover, rats which maintained their weight in the early post-operative period rarely died and soon began to gain weight slowly and steadily. Lastly, rats which were fed chow diets and showed an early post-operative gain in weight soon ceased to grow and then lost weight for a short period before death. In comparison, hypophysectomized rats provided with purified rations, and which began to gain weight, continued to gain slowly for long periods with practically no mortality within the length of our experimental regimen. Furthermore, whenever unexplainable crises resulted in growth plateaus or weight losses during later stages of the experimental period, those hypophysectomized rats receiving chow invariably had a lesser ability to recover from these recessions than the rats fed purified diets. Thus, a close examination of the changes in body weight of individual rats indicated that a strikingly greater and more prolonged life-sustaining stimulation was elicited in hypophysectomized rats by the purified rations than by the chow diets, especially during periods of stress.

In the experiments of Freud and co-workers, and most other researchers, hypophysectomized rats were provided with food *ad libitum*. Very little information is available concerning food habits and the nutritional requirements of hypophysectomized rats or of the effect of different diets on the general health or body structure of

these animals. In contrast to experiments with voluntary food consumption, Samuels, Reinecke and Bauman (1943) tested the premise that hypophysectomized rats might be able to absorb and metabolize more food than they would voluntarily consume by feeding measured amounts of diet to hypophysectomized rats by stomach tube for periods varying from 15 to 54 days.[3] These animals exhibited definite growth of the skeleton and musculature, as shown by increases in body weight and in femur weight. They also stored nitrogen at a rate about one-third that of intact pair fed controls; nitrogen storage was more rapid than skeletal growth but less rapid than the increases in body weight. On the other hand, fat had been stored at a much higher rate than by intact controls and, probably, accounted for a major portion of the body weight increases. Weights of the adrenals, reproductive organs, thyroids, and spleen decreased in size at rates comparable to those observed in hypophysectomized animals fed *ad libitum*.

It is interesting to compare the caloric intake and growth response of our hypophysectomized rats allowed to eat *ad libitum* with those of Samuels *et al.* which were forced to take food in excess of what they desired. In the latter instance, the caloric intake amounted to about 39.6 calories per rat per day, whereas our animals consumed enough ration 100 and 150 to yield an average of only 22.8 and 22.5 calories per day, respectively, during the first 35 days of the experiment. Towards the end of this period, their weights were in about the same range as the force-fed rats. The average food consumption of our intact rats of the same strain in this weight range has been found to be equivalent to 45 calories on which intake they gained between 4 and 5 grams per day. Thus, the force-fed hypophysectomized rats had to ingest amounts of food almost equivalent to what an intact rat of the same weight would voluntarily eat to attain its maximum increase in body weight. The amount of food fed to these hypophysectomized rats was about 75 per cent in excess of what our hypophysectomized rats of the same weight voluntarily consumed in the process of gaining an average of 0.6 gms. per day. The degree of body weight increase observed in operated rats allowed *ad libitum* access to the purified rations would seem to represent a level more nearly approaching the physiological optimum.

The average protein contents of the carcasses of our hypophysectomized rats fed purified rations were lower than the values for in-

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[3] The diet used was composed of a mixture of powdered ovalbumin, lactalbumin, melted butter, corn oil, cornstarch, dextrin, glucose monohydrate, cellu flour, salt mixture, vitamin B-complex, and percomorph oil suspended in water in sufficient amounts that each cubic centimeter of the suspension contained sufficient solids to yield about 3.3 calories upon digestion. The basal composition of this diet was roughly comparable in its carbohydrate, protein and fat distribution to our ration 150 but had 14 per cent of cellu flour in addition. Each day, the hypophysectomized rats were given three feedings of 4 cc. each at 5-hour intervals.

tact rats (Scheer *et al.*, '43) fed comparable diets (15.2 and 14.3 per cent versus 18.7 and 17.9 per cent, respectively). Therefore, it seems probable that the hypophysectomized rats had a slightly lower ability to build protein than the intact rat of the same age. The fact that the protein content of the hypophysectomized rats was maintained at a level so nearly comparable to that of intact rats during a period of growth in which the body weight was slightly more than doubled certainly indicates that a substantial amount of protein synthesis must have occurred. Since carcass analyses were not made at the beginning of the experiment, and since no nitrogen balance studies were made, the actual amount of protein synthesis and the efficiency of protein utilization are not known.

In so far as our data are concerned, there is no evidence that fat metabolism has been altered by hypophysectomy. Unlike force-fed hypophysectomized animals which retained fat at a much more rapid rate than their intact controls, the carcasses of our hypophysectomized rats allowed to feed *ad libitum* did not have a higher fat content than intact rats fed the same diets. This was true even where 45 per cent of the caloric value of the ration was supplied from fat (ration 150). Since the fat content of these animals did not differ significantly from those receiving ration 100, it can be concluded that fats and carbohydrates are utilized as energy sources by the hypophysectomized rats with comparable efficiency.

The values for ash and carbohydrate in the carcasses of the hypophysectomized rats were slightly higher than for intact rats fed comparable rations. It is impossible on the basis of present data to determine whether there is anything of significance about these small differences.

These investigations represent only a beginning in the understanding of the relationship of diet to the survival, growth and body composition of hypophysectomized animals. The unexpectedness of substantial increases in body weight accompanied by tail growth in hypophysectomized rats allowed to eat *ad libitum* made it necessary to obtain data beyond the original purpose of this investigation. Our data needs to be supplemented by carcass analyses of hypophysectomized rats at the time of operation, and after a short period on chow, by determinations of the basal metabolic rate, and by the rate of utilization of the various major food constituents.

## SUMMARY

Between 60 and 70 per cent of rats fed purified diets *ad libitum* after surgical removal of the hypophysis survived for experimental periods of at least 88 and 112 days. Only 10 per cent of comparable rats offered laboratory chow survived beyond 45 days and none more than 63 days. The long survival of hypophysectomized rats fed the purified rations was not due to the fine particle size of the purified

rations nor to their lack of undigestible crude fiber. Those hypophysectomized rats which survived for long periods increased considerably in body weight and, apparently, synthesized considerable protein during the growth process. Tail growth was observed, individual caudal vertebrae lengthened, and the epiphyses of the caudal vertebrae did not close. Hypophysectomized rats which had been maintained on purified rations for 112 days, during which time they had doubled their body weight, had a slightly lower protein content but, otherwise, did not differ greatly in their carcass analyses from intact rats fed purified rations for comparable periods.

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# MODE OF ACTION OF OESTROGENS ON THE CORPUS LUTEUM

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It is well known that oestrogens can maintain the activity of the corpus luteum in the rabbit, in the absence of the pituitary. That such luteal tissue exerts its normal secretory activity is shown by the fact that in non-pregnant animals it produces its typical effect on the endometrium, i.e. progestational proliferation, and in pregnant animals, it maintains a normal gestation (Westman & Jacobson, 1937; Robson, 1937, 1939).

The question arises whether the oestrogens act directly on the luteal cells or whether there is some intermediate mechanism which ultimately results in the effect on the corpus luteum. There is indeed other evidence that oestrogens can exert an action on the ovary (Aron & Aron, 1946) and the results of Bullough (1943) suggest that this effect is a direct one, involving action on the ovarian cells by the oestrogen present in the follicular fluid.

The present experiments were started in the expectation that clear evidence for a direct action of oestrogen on the corpus luteum would be obtained, but in this we have been disappointed. On the contrary, the results are in favour of the view that the effect is indirect. It is possible that this is due to technical deficiencies in our methods, and our data are presented in the hope that they may be of help in other and perhaps more conclusive investigations.

## METHOD

In order to determine the site of action of oestrogens on the corpus luteum, implants of oestrogens were made into a single corpus luteum of pseudo-pregnant rabbits. The animals were made pseudo-pregnant by the injection of pregnancy urine gonadotrophin. Implantations were carried out using "crystal guns" of several sizes depending on the dimensions of the implant. A crystal gun consists of a fine pointed glass tube into which the implant is inserted. The corpus luteum was pierced with the point of the tube and the crystal driven in by means of a well-fitting central rod used as a piston. The animals were hypophysectomized by the method of Firor (1933) on the day after implantation. Macroscopic observations of the condition of the corpora lutea in the two ovaries were made subsequently at one or

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more laparotomies. The animals were finally killed and the two ovaries examined microscopically.

If the implanted oestrogen acts directly on the cells of the corpus luteum it would be expected that shortly after hypophysectomy (about the 4th day) and for some time subsequently, the implanted corpus luteum would be maintained in size and histological development. The remaining corpora lutea of the implanted ovary might show *some* degree of maintenance while the corpora lutea of the unimplanted (control) ovary would be expected to show involution. If, on the other hand, the maintenance of the corpus luteum produced by oestrogen administration is due to an indirect mechanism, e.g. possibly as the result of the formation of another substance acting on the corpus luteum but produced in some other tissue under the influence of the oestrogen, then oestrogen implantation would lead either to some degree of maintenance of the corpora lutea of both ovaries (with adequate dosage) or to failure of maintenance in both ovaries if the dose were inadequate.

## RESULTS

### *Preliminary Experiments on Mice*

As a first step, preliminary experiments were made to determine, for certain oestrogens, a dose small enough to be readily implanted or injected into the rabbit's corpus luteum, which would yet exert an oestrogenic effect for at least 10 days.

The oestrogens used were administered to ovariectomized mice, by subcutaneous injection, subcutaneous implantation or intrarenal implantation of solid material; using the crystal gun in the last two instances. The oestrogenic effect was followed by daily vaginal smears. The condition of the smear was noted by the semi-quantitative

TABLE 1. VAGINAL EFFECT OF CERTAIN OESTROGENS IN OVARIECTOMIZED MICE, WITH DIFFERENT METHODS OF ADMINISTRATION

| Compound | Mode of administration | Dose | No. of animals tested | Duration of response* | |
|---|---|---|---|---|---|
| | | | | Full oestrus, days | Positive responce, days |
| Triphenyl chlorethylene crystals (obtained by slow evaporation of a benzene solution). | Subcutaneous implantation | 0.03–0.49 mg. | 7 | 0–5 | |
| Triphenyl chlorethylene crystals (obtained by slow evaporation of a benzene solution). | Intrarenal implantation | 0.024–0.52 mg. | 8 | 0 | |
| 1% aqueous suspension of oestrone. | Subcutaneous injection | 10–50 μg. | 8 | 0–13 | |
| 1% aqueous suspension of oestrone. | Intrarenal injection | 10–50 μg. | 10 | 0–4 | |
| Oestradiol dipropionate crystals (obtained by cooling a solution in hot alcohol). | Subcutaneous implantation | 16–137 μg. | 12 | 24–55 | |
| Oestradiol dipropionate crystals (obtained by cooling a solution in hot alcohol). | Intrarenal implantation | 14–58 μg. | 6 | 9–45 | |
| Stilboestrol crystals (obtained by cooling a solution in hot alcohol) | Subcutaneous implantation | 5–50 μg. | 10 | 6–30 | |
| [illegible] l by [illegible]hol) | Intrarenal implantation | 5–50 μg. | 10 | 5–66 | |
| [illegible] in cholesterol mixture. | Subcutaneous implantation | 20–60 μg. | 8 | 0–8 | 0–10 |
| 0.1% oestradiol dipropionate in cholesterol mixture. | Subcutaneous implantation | 10–60 μg. | 8 | 0 | 0–7 |

* I.e., the interval between implantation and last day on which the vaginal smear showed complete cornification (full oestrus) or showed partial change (positive response).

method previously described (Robson, 1938) and a smear was judged to be positive as long as it differed significantly from that found in untreated ovariectomized mice. These preliminaries seemed to be essential, since administration of an oestrogen which produces a significant effect in the vagina in the mouse might, when administered into the corpus luteum, be expected to discharge oestrogen into that organ for at least the same period of time.

Renal implantation was used since the corpus luteum is a very vascular structure and it seemed possible that absorption from it might be at a different rate from that which obtains in the comparatively avascular subcutaneous tissues of the mouse. The results are given in Table 1.

It will be seen that oestradiol dipropionate and stilboestrol appeared to be satisfactory, while injections of aqueous oestrone suspension produced effects which were too limited in duration. It should be noted that in experiments with oestrone and triphenyl chlorethylene, intrarenal administration was relatively ineffective as compared to subcutaneous administration (Table 1); while in the case of oestradiol and stilboestrol, comparable effects were produced by both methods of administration. This suggests that oestrone and triphenyl chlorethylene are selectively destroyed or excreted in the kidney, but further investigations on this point are necessary.

As it is known that absorption of steroid hormones is delayed when they are administered as mixtures with cholesterol, experiments were also carried out with implants containing 1% and 0.1% of oestradiol fused with cholesterol. An attempt was made to find a dose which would produce an effect on the vagina less than that of complete oestrus, since it seemed likely that such a dose might produce a very localized effect on the corpus luteum.

Table 1 shows that this result was sometimes achieved with the smaller doeses. The results are expressed as the interval, in days, between implantation and the last day on which full cornification (full oestrus) was observed and also as the interval, in days, up to the last day during which a definite but partial effect, short of complete cornification was observed (positive response).

### *Experiments on Rabbits*

*Oestradiol dipropionate implants:* (see Table 2) Eight complete experiments with this substance in doses of 5, 10 and 18 $\mu$g. were carried out. Experiments thus reported as complete (here, as well as below) are those in which (1) macroscopic examination of the sella turcica at post mortem showed that hypophysectomy had been complete and (2) the period of survival of the animal after the operation was adequate for the purposes of the experiment, as outlined above.

The results of these experiments are summarized in Table 2. It will be observed that they fall into three groups. In the first group, in

one animal only, the corpora lutea were not maintained to the end of the experiment. The results are interesting as they show that the corpora lutea degenerated at about an equal rate in both ovaries. On the 10th day after hypophysectomy examination of the corpora lutea in both ovaries, examined at laparotomy, showed full maintenance; at the 16th day macroscopic examination suggested that some degeneration was already occurring and affecting both ovaries equally. When the animal was killed on the 25th day, the corpora lutea in both ovaries were fully degenerated. In the second group, there was partial maintenance of corpora lutea in both ovaries. This

TABLE 2. EFFECT OF OESTROGEN IMPLANTS INTO A SINGLE CORPUS LUTEUM OF RABBITS HYPOPHYSECTOMIZED ONE DAY AFTER IMPLANTATION

| Substance and dose implanted (μg.) | Interval between hypophysectomy and death | Degree of maintenance of the corpora lutea |
|---|---|---|
| *Oestradial Dipropionate* | | |
| 5 | 16 | Full in both ovaries |
| 5 | 6 | Partial in both ovaries |
| 10 | 15 | Full in both ovaries |
| 10 | 25 | None in either ovary |
| 10 | 11 | Partial in both ovaries |
| 10 | 23 | Full in both ovaries |
| 18 | 7 | Full in both ovaries |
| 18 | 7 | Full in both ovaries |
| *Stilbestrol* | | |
| 5 | 4 | Partial in both ovaries |
| 5 | 6 | Full in both ovaries |
| 15 | 6 | None in either ovary |
| 15 | 7 | Full in both ovaries |
| 15 | 4 | Partial in both ovaries |
| *Oestradiol 1% in Cholesterol* | | |
| 60 | 5 | Partial in both ovaries |
| 60 | 7 | Full in both ovaries |
| 60 | 7 | Full in both ovaries |

was observed in two animals 6 and 11 days after hypophysectomy respectively. In these, the corpora lutea had undergone some shrinking and there was some invasion by leucocytes. In the third group, there was full maintenance of the corpora lutea in both ovaries. This was observed in five cases, for periods ranging from 7 to 23 days after hypophysectomy.

*Stilboestrol Implants* (see Table 2): Five complete experiments with doses of 5 or 15 μg. of the crystalline substance were carried out. In two cases, corpora lutea were fully maintained in both ovaries 6 and 7 days after hypophysectomy respectively. In two cases, partial maintenance was observed in the two ovaries 4 days after hypophysectomy. One case showed failure of maintenance 6 days after hypophysectomy.

*Implants of 1% Mixture of Oestradiol Dipropionate in Cholesterol:*

Three experiments using 60 $\mu$g. of the mixture were completed (see Table 2). In two animals, full maintenance of the corpora lutea was observed on the 7th day after hypophysectomy. In one case, partial maintenance only was shown 5 days after hypophysectomy.

It will be noted that no selective effect on the corpora lutea of the implanted ovary was obtained in any of these experiments. Even when the maintenance effect was incomplete, the corpora lutea of both ovaries were preserved to the same extent. Hence the fact that a selective effect of the oestrogen was not observed, cannot be explained on the assumption that the dose necessary to produce a local effect only is very critical i.e. that a dose little above it will maintain the corpora lutea in both ovaries equally, while a dose little below it will not maintain any corpoa lutea.

## DISCUSSION

The above results indicate that under the condition of the experiments, the corpus luteum, with oestrogen implanted in it, was maintained to the same extent as the corpora in the control ovary. In most cases, the corpora of both ovaries were maintained equally; slight differences in luteal development in the two ovaries were clearly not significant. In a minority of cases the results fell into equal groups, showing (a) partial maintenance of the corpora lutea in both ovaries or (b) complete failure of maintenance of the corpora at the time of conclusion of the experiment.

Although the problem cannot be regarded as settled, these results suggest that the maintenance effect of oestrogen on the corpora lutea of hypophysectomized animals is an indirect one. This presents certain difficulties in view of the results of previous work (Bullough, 1943). Nevertheless, the present findings are compatible with the view that oestrogens act directly (and as such) on the cells of the corpus luteum, only if it is accepted that oestrogen locally administered does not diffuse into the cells of the corpus luteum from the site of implantation but is entirely absorbed, at or in the immediate vicinity of this site, directly into the circulation. The highly vascular nature of the organ certainly lends some support to this suggestion, though it must be remembered that local effects have been produced in other highly vascular organs, e.g. by the implantation of androgen into the testis (Dvoskin, 1944; Smith, 1944) and of thyroid stimulating substance into the thyroid (Aron & Aron, 1947).

It should be added that in an attempt to find a decisive answer to the problem, certain other experiments were devised. Transplants of the ovary into the spleen were made in a few animals, but attempts to produce luteinization of the follicles of the transplanted ovaries failed. If the technical difficulties of this experiment could be surmounted, the effect of the implanted oestrogen could be confined to the implanted ovary, as any oestrogen absorbed into the blood stream would

now enter the portal circulation and be inactivated in the liver. Hence if maintenance of the corpora lutea in the implanted ovary under such conditions could be obtained, the response would not be attributable to the effect of circulating oestrogens.

## SUMMARY

Small doses of various oestrogens were implanted as crystals or injected as aqueous suspensions into mice to find those which would produce a prolonged oestrogenic effect in amounts suitable for introduction into a rabbit's corpus luteum.

Crystals of suitable oestrogens were implanted into one corpus luteum of pseudo-pregnant rabbits and the animals were then hypophysectomized. In no animals were the implanted corpora maintained selectively. The corpora lutea in both ovaries were either equally maintained, or degenerated at about the same rate. This evidence suggests that oestrogens do not act directly on the luteal cells but through some indirect mechanism.

## ACKNOWLEDGMENTS

The expenses of this investigation were in part defrayed by a grant from the Medical Research Council (to J. M. R.). We are indebted to Organon Ltd. for the supply of oestradiol and urinary gonadotrophin and to Parke Davis Ltd. for the oestrone suspension.

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RESULTS

The time necessary for a positive response to occur in the test animals was found to vary indirectly and as a hyperbolic function of the size of the dose administered when between 22.5 and 100 I.U. of gonadotrophin was used. At these dosage limits the curve became asymptotic. The mean deviation of the animals at each dosage level was such that 4 animals were requisite to an adequate analysis at any

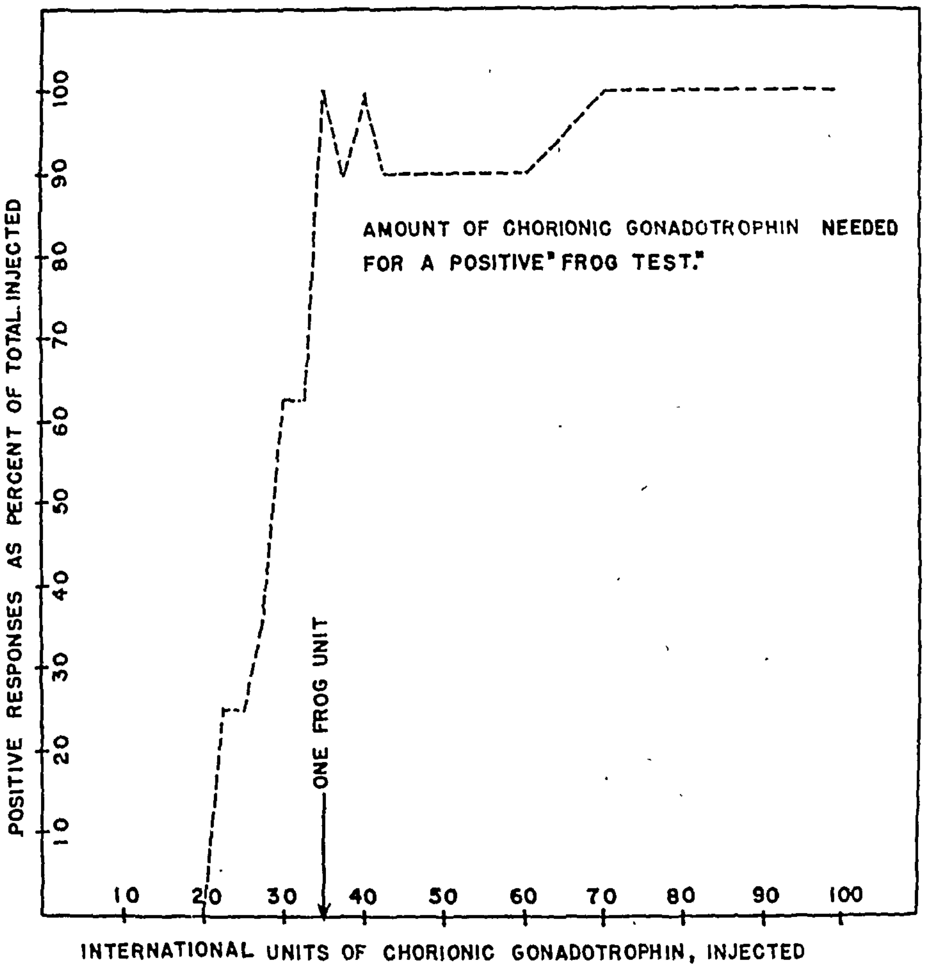


FIG. 2.

given level. An examination of the curve further reveals that at doses between 35 and 60 I.U., estimation of the dose of gonadotrophin from the time required for a positive response is most accurate. Figure 1 is offered as a graphic illustration of these findings.

Figure 2 indicates the response of the test animals to various amounts of intraperitoneally injected chorionic gonadotrophin without regard for the time element, but only in terms of positiveness or negativeness. At 70 I.U. and above, 100% of the test animals yielded

urinary sperm. From 35 to 60 I.U. inclusive, the animals responded with only an occasional negative result. The occasional negative response found in these dosages illustrates the necessity of utilizing two animals in routine "pregnancy testing" and the resultant increase in accuracy as compared to those tests that are made using only one test animal.

Below 35 I.U. of gonadotrophin the percentage of positive responses rapidly decreased and at the level of 20 I.U. and below, the presence of urinary sperm could no longer be detected. As a result of these findings, a male Rana pipiens "frog unit" of gonadotrophin is defined for use in this laboratory as the smallest amount of chorionic gonadotrophic substance necessary to cause a positive response in 3 of 4 test animals. This unit approximates 35 I.U. of chorionic gonadotrophin, which compares favorably with the minimal effective dose of gonadotrophin for a positive response in the male Bufo arenarum Hensel of 40 I.U. (Galli-Mainini, 1948).

When determining gonadotrophic titre in this laboratory an attempt is made to locate two points on the time response-dose curve, Figure 1, by dilution of the test substance and repeated testing with groups of 4 frogs. Calculation then gives the gonadotrophic titre of the substance tested expressed as I.U. or frog units. The estimated titre may further be verified, if desired, by calculating the amount of the test substance equivalent to 35 I.U. and testing of an appropriate number of animals at this level and below for the minimal effective dose.

## SUMMARY

The male Rana pipiens reacts to chorionic gonadotrophin by liberating sperm into the urine. At least 35 I.U. of gonadotrophin are necessary to provide a positive response in 75% of these animals, whereas doses of 70 I.U. and above initiate a positive response in 100% of tested animals. The time required for the liberation of sperm is indirectly proportional to the dose of gonadotrophin administered. The time response-dose relationship is constant enough to provide a relatively accurate quantitative bio-assay technique which is complemental to the conventional minimal effective dose technique.

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# COMPARATIVE ACTIVITY OF THIOURACIL AND OTHER ANTITHYROID COMPOUNDS IN THE RHESUS MONKEY

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## INTRODUCTION

THE selective iodine concentrating mechanism of the thyroid gland has been clearly demonstrated to occur in two independent steps (Vander Laan and Vander Laan, 1947) (Taurog, Chaikoff and Feller, 1947). The first step which involves absorption of iodide ion from the blood is not inhibited by antithyroid agents of the thiouracil type and accounts for but a small portion of the total iodine present in the gland at any time. The second step involves the conversion of absorbed iodide into organically bound form, a considerable portion of which is of hormonal character (Wolff and Chaikoff, 1947). This mechanism accounts for the greater portion of accumulated iodine of the normal gland and is inhibited by thiouracil-like antithyroid drugs. On the basis of these considerations, McGinty and collaborators (1948) quantitated the activity of several antithyroid agents in rats and chicks following a single subcutaneous injection of the compounds in varying doses. The duration of inhibition of hormone synthesis as evidenced by block to iodine collection was determined by administration of tracer doses of radioactive iodine at intervals following administration of drug, iodine absorption being followed by radioactivity counts on excised glands. In the rat, the antithyroid potency of the compounds studied paralleled in a general way the activity of the same substances as estimated by the 10 day chronic feeding test (Astwood, Bissell and Hughes, 1945; McGinty and Bywater, 1945). In the chick this parallelism did not hold. The duration of action of minimal effective doses in both rat and chick was not materially different, escape from the blocking action occurring at 8 hours in the rat and 6 hours in the chick.

Using the same general principle, Stanley and Astwood (1947) assayed 32 compounds for antithyroid activity in man. Following administration of radioactive iodine, radioactivity counts were recorded with a shielded G-M counting tube placed over the sternal notch until such time that an individually characteristic "accumulation gradient" or rate of iodine uptake by the thyroid was established. At this time,

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usually from one to two hours after radioiodine administration, varying amounts of the compounds under study were administered orally. Potency was estimated from the degree and duration of alteration of the accumulation gradient slope.

This important investigation brought out two facts, one, that the antithyroid activities of the compounds evaluated in man were in most instances quite different from the potencies as estimated from rat and chick experiments, and, two, that these activities appear to correspond more closely to results obtained by clinical experience in treatment of thyrotoxicosis rather than what would be expected from rat or chick experiments.

It is the purpose of this communication to present similar comparative studies on a few compounds in the monkey. The drugs studied included thiouracil and the 6-methyl, 6-propyl and 6-benzyl derivatives, 2-mercapto-5-amino-thiadiazole (TC-68), 3-(phenylaminomethyl)-2-thiazolidinethione (TC-105), 2-mercaptoimidazole, 2-mercaptoimidazoline, 2-mercaptothiazole and 2-mercaptothiazoline. The last four compounds were of particular interest because of the high antithyroid potencies of 2-mercaptoimidazole and 2-mercaptothiazoline reported by Stanley and Astwood in man—10 and 2.5 times that of thiouracil respectively.

## METHOD

One female and nine male rhesus monkeys of 4–7 kilograms body weight were housed singly in roomy screen-bottom cages in an air-conditioned room. They were given a rotated diet, *ad libitum*, of cooked cereals, fresh fruits and vegetables, bread, peanuts and milk. In addition, each animal received 1.2 ml. of a liquid vitamin supplement (Abdec) daily on a sugar cube.

At the outset of each experiment, which was repeated at monthly intervals, carrier-free $I^{131}$ was administered intravenously in sterile saline at doses of approximately 20 microcuries. Radioactivity counts were taken a few minutes following radioiodide injection and at one-half hour intervals for three hours at which time the antithyroid drug under study was administered. Counts were then taken at hourly intervals until the seventh hour and again at the 11th, 24th and 48th hour from radioiodine injection.

Counting was done by means of a 6 cm. diameter shielded Victoreen tube and a Tracerlab Autoscaler. Records were taken over the midline of the neck in the angle between the clavicles and suprasternal notch. The G-M counter was checked at least twice daily against a radiocobalt standard. Antithyroid drugs were administered in gelatin capsules inserted manually into the posterior pharynx.

## RESULTS

In conformity with the results reported by Stanley and Astwood for man, we found also that the rate of radioiodine uptake by the thyroid of the monkey as measured by the externally positioned counter, when plotted against time, forms a distorted S-shaped curve,

the greater curvature of which is parabolic in shape. When radioactivity counts are plotted against the square root of the elapsed time in minutes, the major portion of the curve, after the first half hour and for at least 11 hours, was essentially linear. This is shown in Figure 1 in untreated animals in which individual neck counts are plotted against the time at which they were recorded. Repetition of these control experiments indicated that the slope of the iodine col-

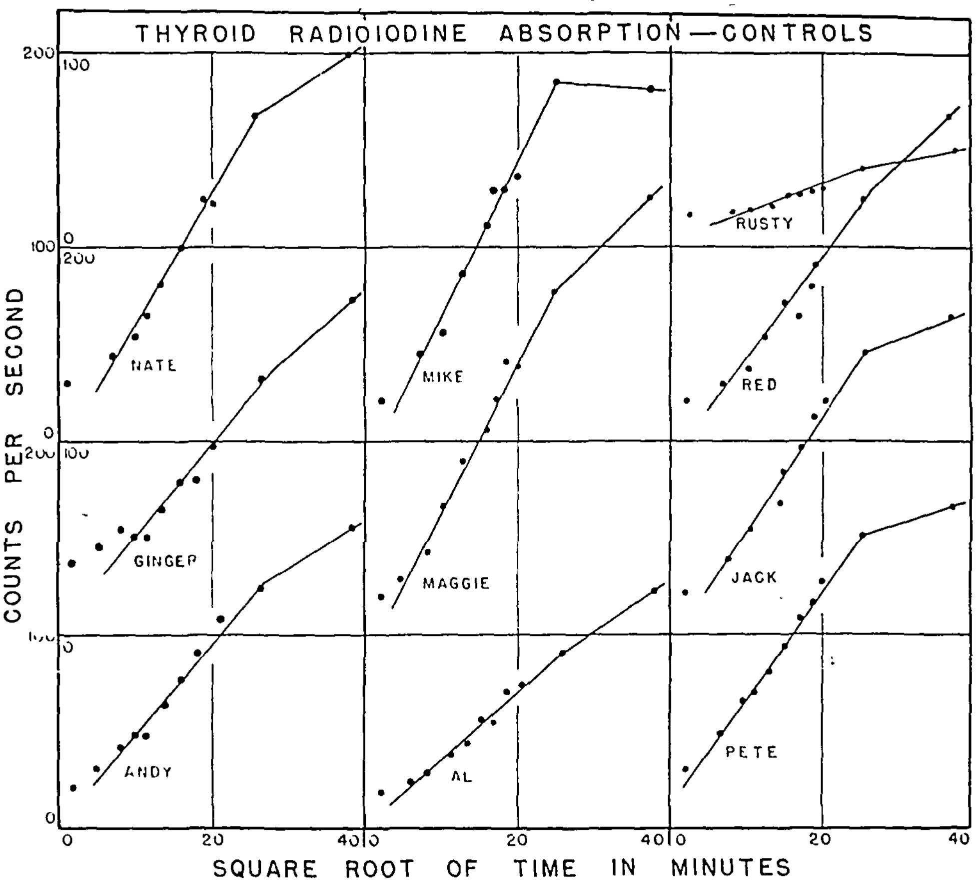


FIG. 1. Showing uptake of radioactive iodine by the thyroid of ten normal Rhesus monkeys following intravenous injection of 20 microcuries carrier-free radioiodide ($I^{131}$). Radioactivity in the thyroid, taken from Geiger-Mueller counter measurements over the suprasternal notch, is plotted against the square root of the time in minutes from radioiodide injection. The straight line through the individual points on the graph is indicative of the rate of accumulation of iodine by the thyroid over the time interval measured and of the corresponding rate of hormone synthesis.

lection curve varied between different monkeys and in the same monkey at different times. It was necessary, therefore, that estimations of potency of administered drugs be made on deviations from the curve, the slope of which was established immediately preceding administration of the compound under study. Because of the persistently flat curve in the case of Rusty, experiments on this animal were not undertaken.

Experimental results with thiouracil and the three derivatives of thiouracil investigated are shown in Figure 2. The initial rate of iodine collection is plotted as a straight line from neck count data taken at half hour intervals beginning one-half hour after injection of radioiodine and extending to the end of the third hour, when the drug was administered. This slope is extended as a broken line to the 12th

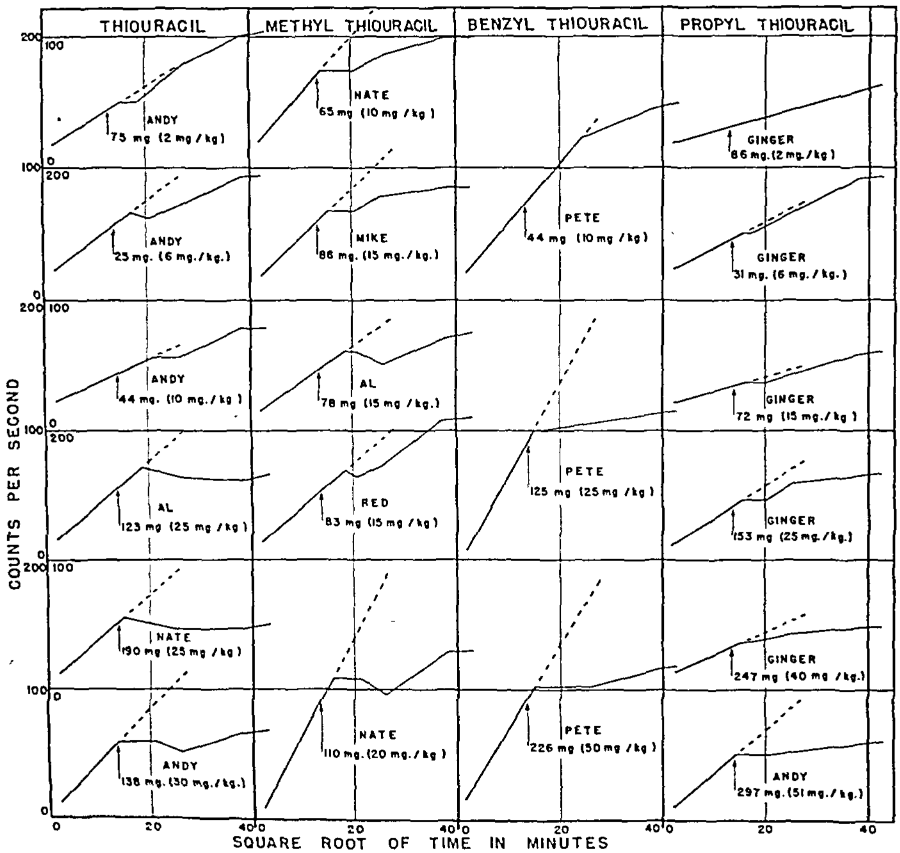


FIG. 2. Showing deviations from the established rate of uptake of radioiodine by the thyroids of monkeys when given varying doses of thiouracil and three of its 6-substituted derivatives. The solid portion of the curves represent the accumulation slope of iodine uptake as plotted from radioactivity measurements taken in the first three hour period following radioiodide injection and after oral administration of the antithyroid compound at the beginning of the fourth hour. The broken line indicates anticipated radioiodine uptake for an 11 hour period had no drug been given. Full antithyroid activity is indicated by no increase in radioactivity over the thyroid region.

hour ($t^{\frac{1}{2}}_{min} = 25.5$) to indicate expected radioiodine absorption had no drug been given.

A dose of 7.5 mg. thiouracil, equivalent to 2 mg. per kg. body weight, exerted only a brief inhibitory action on iodine uptake, the curve of accumulation of radioiodine by the thyroid resuming a pattern practically the same as that expected from the untreated animal.

Twenty-five milligrams of thiouracil in Andy was more effective and persisted for about two hours when iodine collection was resumed at a somewhat reduced rate. This reduction in rate of radioiodine uptake was a usual occurrence following temporary inhibition and may have been due to loss of radioiodine through the kidneys during the non-absorptive period. A larger dose of 10 mg. per kg. in Andy induced an inhibition of no more than four hours duration. Administration of thiouracil in amounts equivalent to 25 and 30 mg. per kg. in Al, Nate and Andy were fully effective for 24 hours or longer. Any attempt to evaluate the duration of action of the drugs beyond 24 hours from the 48 hour radioactivity count was invalidated by lack of information on the fate of injected radioiodine during the thyroid inhibition period.

Results with methyl thiouracil were variable. Doses of 10 and 15 mg. per kg. were effective for periods varying from one to four hours although, with the exception of Red, some reduction in the rate of radioiodine absorption by the thyroid was evident for 24 hours. Twenty milligrams per kilogram methyl thiouracil was fully inhibitory for 8 hours, following which iodine absorption was resumed at almost the initial rate. The number of experiments with methyl thiouracil were too few to evaluate comparatively the antithyroid activity of this compound with thiouracil, but it does not appear to differ from the latter substance materially. This is consistent with clinical experiences with this drug.

Benzyl thiouracil inhibited iodine collection by the thyroid in two experiments with 25 and 50 mg. per kg. It was wholly inactive at a 10 mg. dose in Pete, the trend of iodine absorption away from the projected gradient being considered of no significance since it occurred 12 hours following administration of the drug. Benzyl thiouracil was estimated by Stanley and Astwood to be 0.75 times as active as thiouracil on the basis of their thyroid radioiodine absorption experiments in man. Monkey experiments, while too few to provide quantitative data, indicate that this compound is at least in the same order of activity as thiouracil.

Propyl thiouracil did not wholly inhibit radioiodine collection in doses up to 40 mg. per kg. body weight. In a single experiment, a dose of 51 mg. per kg. caused an iodine block lasting for 24 hours. These results conform in a general way with those of Stanley and Astwood which indicated that propyl thiouracil was less active than thiouracil.

2-Mercaptoimidazole was reported by Stanley and Astwood to have an antithyroid activity at least 10 times that of thiouracil. On the basis of similar experiments in monkeys, we estimated that this compound was 20 or more times as active as thiouracil. It will be observed from Figure 3, that a dose of 0.9 mg. or 0.2 mg. per kg. in Nate was only slightly inhibitory to radioiodine collection. However, 0.6 mg. per kg. in the same monkey produced a complete block to iodine

uptake for a period of approximately eight hours. In three other experiments, doses of 1.0 mg. per kg. were completely inhibitory for periods up to 24 hours. These results are comparable to those obtained with 25 mg. per kg. of thiouracil.

2-Mercaptoimidazoline, the saturated analog of the highly active 2-mercaptoimidazole, was found to be fully inhibitory to radioiodine

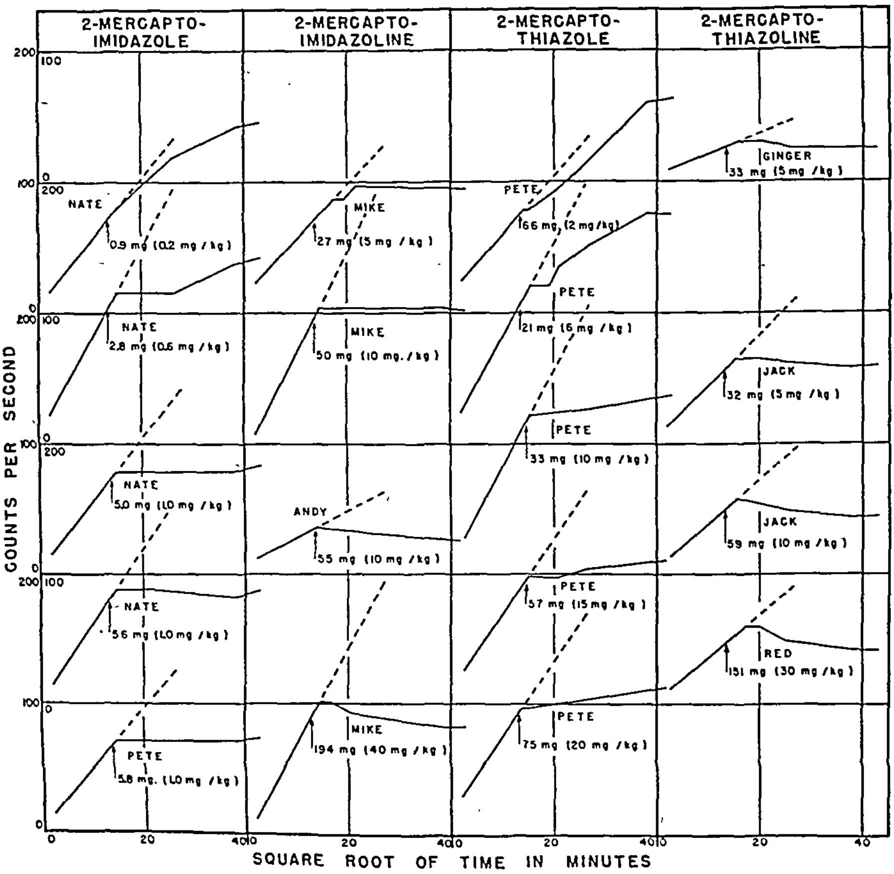


FIG. 3. Showing alterations in the accumulation gradient of radioiodine by the thyroids of monkeys given varying amounts of 2-mercaptoimidazole, 2-mercaptoimidazoline, 2-mercaptothiazole and 2-mercaptothiazoline.

absorption for a period of at least 24 hours in doses of 10 mg. per kg. and suggestive in a single monkey of prolonged inhibition in a dose of 5 mg. per kg. On a comparative basis, this substance was estimated to be at least twice as active as thiouracil, a finding contrary to that reported by Stanley and Astwood. These workers rated 2-mercaptoimidazoline half as active as thiouracil in man.

2-Mercaptothiazole and 2-mercaptothiazoline, the sulfur analogs of 2-mercaptoimidazole and 2-mercaptoimidazoline, were studied in

monkeys. 2-Mercaptothiazoline was estimated by Stanley and Astwood to possess 2.5 times the activity of thiouracil. Our experiments in monkeys were incomplete in that the minimal inhibitory dose of this compound was not determined. Administration of 5 and 10 mg. per kg. blocked iodine collection by the thyroid for 24 hours or more and by comparison was estimated to be more than five times as active as thiouracil, an appraisal higher than the value of 2.5 assigned by the Boston workers. 2-Mercaptothiazoline was evaluated for antithyroid activity by the 10-day chronic feeding test in rats (Astwood, Bissell and Hughes, 1945; McGinty and Bywater, 1945) and results from both laboratories were in agreement that this compound was 1.3 times as active as thiouracil. It is apparent that its activity in the human and in monkeys is comparatively much greater.

On the basis of structural relationship and relative antithyroid activities in both man and monkey of 2-mercaptoimidazole and 2-mercaptoimidazoline on one hand and of 2-mercaptothiazole and 2-mercaptothiazoline on the other, it was expected that 2-mercaptothiazole might possess extremely high antithyroid activity compared with thiouracil. It was found, however, that doses of 10 mg. per kg. were required to maintain the thyroid in an inhibited state for 24 hours. The compound is estimated to be about twice as active as thiouracil.

2-Mercapto-5-aminothiadiazole (TC-68) and 3-(phenylaminomethyl)-2-thiazolidinethione (TC-105), two compounds investigated extensively in rat and chick experiments, were found in monkeys to be approximately twice as active as thiouracil. In the case of TC-68, this value agreed with that reported by Stanley and Astwood. These investigators did not test TC-105.

## DISCUSSION

Estimations of antithyroid potencies of various compounds on the basis of inhibition of radioiodine collection by the thyroid after injection of carrier-free radioiodide appear valid. Any significant absorption of iodine by the thyroid from blood whose iodide concentration has not been altered materially may be considered as a measure of and directly proportional to hormonal iodine formation by thyroid cells. Thus, any reduction in the rate of absorption of iodide whether measured directly or by the tracer technic may be considered due to decreased hormone synthesis and a quantitative measure of antithyroid potency of most substances whose administration produces this effect. In this connection, it must be pointed out that Vander Laan and Vander Laan (1947) have shown that the goitrogenic action of KSCN is due not to inhibition of protein binding of iodine and hormone synthesis but to interference with entry of iodide into thyroid cells.

Stanley and Astwood call attention to certain limitations in the radioiodine absorption method as a measure of clinical effectiveness of

certain antithyroid compounds. They point out that the procedure does not distinguish between compounds relatively weak by the test but clinically effective because of cumulative action and those highly active inhibitory agents which may be clinically ineffective because of rapid elimination and non-cumulative properties. The same objection applies to the findings on monkeys. Examination of the curves on propyl thiouracil and benzyl thiouracil, which according to Stanley and Astwood are short-acting antithyroid agents, indicates that these drugs, even in the larger doses, are not completely inhibitory. This may be due to rapid kidney loss, but in human subjects we have shown (McGinty, Sharp, Dill and Rawson, 1949) that propyl and benzyl thiouracil are, in fact, eliminated at the same rate as thiouracil and methyl thiouracil. In contrast, however, to the latter substances, propyl and benzyl thiouracil appear in the urine in acid-hydrolyzable form. We have speculated that this conjugation may account for the clinical ineffectiveness of benzyl thiouracil and to a lesser extent for that of propyl thiouracil relative to the high antithyroid potency of these compounds in rats and chicks. Whether or not conjugation occurs in the monkey, we do not know.

The monkey appears to be considerably less sensitive to antithyroid agents than man. Comparison of available data indicates that 5 to 10 times as much drug is required to produce comparable thyroid inhibitory effects in the monkey.

On the whole, our estimation of relative antithyroid activities from the monkey experiments agreed with those conducted by a similar procedure in human subjects. The only noteworthy exception was in the case of 2-mercaptoimidazoline which we rated as twice as active as thiouracil but to which Stanley and Astwood assigned a value of 0.5. Both laboratories agreed as to the extremely high potency of 2-mercaptoimidazole and it is hoped that further clinical information on this interesting drug will be forthcoming.

It was noted that control accumulation gradient curves from serial experiments in the same animal were invariably less steep than preceding curves. What caused this is not known. Rough calculations from neck counts, estimated geometry, etc. indicated that three-quarters or more of the administered dose of radioactive iodine was taken up by the thyroid within 48 hours. Further crude estimates revealed that this absorption may have amounted to 50 or more microcuries per gram of thyroid tissue. According to the recent extensive work of Haines, *et al.* (1948), this dose of radiation over the whole decay period approaches that which caused definite reduction in mass of thyroid tissue and fall in B.M.R. in thyrotoxic patients.

## SUMMARY

Estimations of antithyroid activity of a group of compounds were carried out in normal monkeys after intravenous administration of radioactive iodine by observing the degree and duration of alteration

of an established rate of accumulation pattern by the thyroid gland. The antithyroid drugs were administered orally and radioactivity in the thyroid was recorded by external counting.

Assigning thiouracil an evaluation of 1.0, the following results were obtained: 2-mercaptoimidazole, 20; 2-mercaptothiazoline, >5; 2-mercaptoimidazoline, 2; 2-mercaptothiazole, 2; 2-mercapto-4-aminothiadiazole, 2; 3(phenylaminomethyl)-2-thiazolidinethione, 2; methyl thiouracil, 1; benzyl thiouracil, 1; and propyl thiouracil, 0.5. These values are in general agreement with estimations similarly obtained in man by Stanley and Astwood.

## ACKNOWLEDGMENTS

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# A NOTE ON EPITHELIAL METAPLASIA IN THE MALE GENITAL TRACT[1]

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ATTEMPTS have been made by several investigators to study various instances of metaplasia of sex accessory epithelia and to arrive at a possible common etiologic factor for all such metaplasia. On the basis of somewhat inconclusive data, McCullough and Dalldorf (1937) suggested that vitamin A deficiency was a prerequisite for all true epithelial metaplasia. Hume, Burbank, and Korenchevsky (1939) produced identical lesions in male rat sex accessories by deprivation of vitamin A and by administration of large amounts of estrogen. Whether such identical lesions occurred in non-genital tissues was not fully indicated. Cramer (1942) obtained similar results from experiments on the rat uterus.

Kasman and Gold (1933) and Sutton and McDonald (1943) studied metaplasia in the human prostate, and the latter concluded that benign metaplastic changes following transurethral resection were due to "the basic polymorphism of epithelial cells" and their reaction to necrotic and regenerative processes following the resection.

In the course of a series of studies involving the experimental production of sex accessory pathologies in the male Dutch rabbit, several instances of epithelial metaplasia were noted. The effect of short and long treatments with estrogens in inducing a non-keratinized, non-squamous, stratified columnar ("transitionoid") metaplasia has already been reported in detail (Bern, '49a, b). An entirely different type of epithelial metaplasia, hitherto unreported, was observed in a large number of animals employed during the course of these studies. Keratinized nodular lesions were found in the prostate of 45% and in the vesicular gland of 71% of a total of 80 normal and experimental rabbits. In no instance were these lesions found in the seminal vesicle.

The lesions consist of groups of closely packed epithelial cells with large nuclei and undifferentiated cytoplasm and show frequent mitotic figures (figs. 1–2). They originate as dedifferentiated or un-

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differentiated clusters of cells in the alveolar walls in both the prostate and vesicular gland and proliferate under the epithelium into the stroma in much the same way as a neoplastic growth. The cells of the lesion bordering on the lumen of the gland are stratified squamous and show some degree of keratinization, often quite extensive (fig. 2). The adjacent epithelium sometimes undergoes squamous metaplasia and contributes to the keratinization. Occasionally, this latter process is accompanied by leucocytic infiltration.

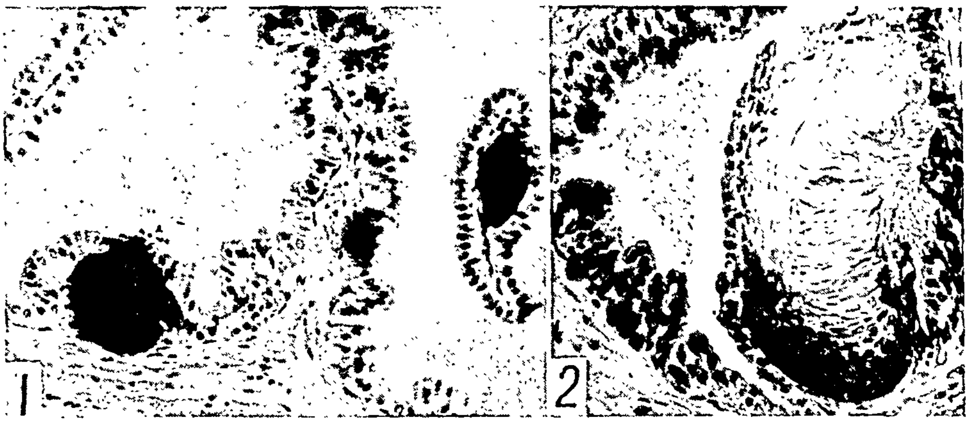


FIG. 1. Two lesions from vesicular gland of untreated rabbit. Lesion on right is compressed between alveolar walls. H. &. E. ×150.
FIG. 2. Keratinized lesion showing involvement of large segment of alveolus in vesicular gland of untreated rabbit. H. & E. ×150.

The lesions are histologically indistinguishable from those described as due to avitaminosis-A in epithelial tissues, including those of the sex accessories, of other animals (Wolbach and Bessey, 1942) and occurred "spontaneously" in our series of Dutch rabbits. Rabbits have need of relatively more vitamin A in their diet than the rat and the pig, and possibly fowl, as was early demonstrated by the experimental production of xerophthalmia (Nelson, Lamb, and Heller, 1922). The histology of avitaminotic-A lesions in the rabbit evidently has not been recorded in the literature, but gross changes indicate similarity to the picture seen in other animals (Wolbach, 1937).

Xerophthalmic indications of avitaminosis-A were incidentally noted in several of our rabbits, along with the low fertility of the breeding stock. Microscopic histologic sections of submaxillary salivary glands and pancreas, as well as other urogenital organs, have been made from time to time, however, and except for rare epididymidal keratinization reported elsewhere (Bern, 1949b), no other histologic evidence of possible vitamin A deficiency has been found in any of the animals examined. However, the recent work of Wilson and Warkany (1947) has indicated the special sensitivity of urogenital

sinus derivatives (including the prostate complex) to lack of vitamin A in the embryonic rat.

To test the adequacy of our rabbit food pellets (a standard commercial "complete" diet), spectrophotometric analyses for vitamin A and carotene content were carried out, and 0.0012–0.0014% carotene was found. The true biologic potency is probably considerably less. Cod liver oil with high vitamin A content was administered to determine if the occurrence of the lesions could be prevented. Of 6 mature rabbits receiving about 10 ml. of oil poured over their food pellets once a week for 2 to $5\frac{1}{2}$ months, three individuals showed lesions regardless of duration of oil administration. Another series of 9 immature (three months of age) male Dutch rabbits was divided into two groups (separating littermates whenever possible): one group of 4 received one ml. of cod liver oil orally by means of a syringe three times weekly for 22 weeks; the second group of 5 served as controls and received the standard diet without cod liver oil. All 9 rabbits upon histologic examination after sacrifice showed lesions in their vesicular glands, although none were seen in the prostates (only sample sections of each gland were prepared).

No conclusions can be drawn as to the adequacy of vitamin A and its precursors in the diet. However, cod liver oil containing vitamin A is evidently unable to prevent or alleviate these lesions in the dosages employed. The cause of the spontaneous lesions in the rabbit, thus, remains undetermined.

In addition to the stratified columnar metaplasia occurring in virtually all male rabbit sex accessories after prolonged estrogenization and the keratinized, stratified squamous spontaneous lesions described above, several other instances of epithelial metaplasia have been noted. These include:

(1) Squamous metaplasia with near-cornified surface cells (due to compression) of vesicular gland epithelium in contact with surgical sutures, distinct from the keratinized nodular lesions; may also result from early contact with methylcholanthrene or paraffin pellets.

(2) Stratified columnar metaplasia of seminal vesicle surface epithelium in contact with voluminous blood clots.

(3) Stratified columnar metaplasia of prostate epithelium due to post-operative infection; accompanying atrophy.

The distinct differences between the spontaneous pathologic lesions (keratinized nodules) and the other types of metaplasia observed lead us to believe that not all metaplasia in the male rabbit urogenital tract is due to the same basic etiologic factor. The prostate complex obviously has at least temporary ability to produce keratinized squamous metaplastic epithelium, but does not so respond under the influence of estrogen. It is possible, however, that the keratinized nodular lesions are established in the younger tissues and that this special reactivity is lost with age.

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# THE EFFECT OF ADRENAL CORTEX EXTRACT WITH AND WITHOUT EPINEPHRINE UPON THE WORK OF ADRENALLY INSUFFICIENT RATS

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EPINEPHRINE has the property of temporarily increasing the height of contraction of "fatigued" skeletal muscle. Radwanska (1910) found that the administration of epinephrine to the "fatigued" adrenalectomized animal gives a temporary increase in work output. Although the hormones of the adrenal cortex are of primary importance in restoring the ability of the adrenalectomized animal to work (Hartman, Brownell and Lockwood, 1932), the question of the importance of the adrenal medulla and its hormone epinephrine in the maintenance of vigor and resistance has remained unanswered. Ingle and Lukens (1941) found that the work performance of the adrenal demedullated rat was depressed below that of normal animals during the first few hours of work and that the administration of epinephrine with glucose would temporarily restore to normal the work rate of "fatigued" adrenalectomized rats. One of us (D.J.I., unpublished) has observed that the continuous subcutaneous injection of adrenal cortex extract with epinephrine to adrenalectomized rats in collapse and near death can cause a reversal of symptoms and recovery which are not obtainable with adrenal cortex extract alone. Recant *et al.* (in press) have observed a synergistic effect of epinephrine and adrenal cortex extract in suppressing the eosinophil count in patients with Addison's disease. It has remained difficult to restore either adrenally insufficient animals or patients to full normal vigor by the administration of cortical extracts and steroids. The possibility that removal or destruction of the adrenal glands creates a deficiency of epinephrine or other principles of the adrenal glands which may be required for normal vigor has been only partially tested by the present studies.

In this study we have tested the possibility that the work performance of the adrenally insufficient rat can be improved to a greater extent by the administration of epinephrine and adrenal cortex extract than by the administration of an equal amount of adrenal cortex extract alone. The results were negative.

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## METHODS

Male rats of the Sprague-Dawley strain which weighed 200±2 grams were used. The diet was Archer Dog Pellets. The procedures for the stimulation of muscle were according to Ingle (1944) with the following modifications. A Nerve Stimulator, Model B. Upjohn, was used to stimulate muscle at the rate of five times per second by a DC pulse of 20 milliamperes having a duration of 20 milliseconds. An electrode was placed on the lower tibia of the left back leg and the second electrode on the contralateral back foot, thereby activating all of the musculature of both hind legs. The gastrocnemius muscle of the left hind leg was weighted with 100 grams. The distance that the weight was lifted was registered on automatic work recorders. Each recorder revolution represented approximately 400 gram-centimeters of work.

A solution of 0.9 per cent sodium chloride containing 1 mgm. of ascorbic acid per cc. and varying concentrations of adrenal cortex extract (ACE) and epinephrine was infused into the jugular vein by means of a constant injection apparatus which delivered fluid from each of 12 syringes at the rate of 20 cc. in 24 hours. In Experiments 2 and 3, glucose (C. P. Merck) was added to the infusion solution in amounts of 200 mgm. per 100 grams of rat per hour. This load of glucose prevented the hypoglycemia which otherwise developed in the working rats. The adrenal cortex extract represented 40 grams of beef adrenal glands per cc. and was free from alcohol. The ascorbic acid acted as an anti-oxidant which effectively preserved the epinephrine.

Our standard procedure for the intravenous administration of solutions was to insert a short bevel 22-gauge hypodermic needle through the pectoral muscle into the jugular vein in a cephalad direction. This procedure was used in Experiments 1 and 2. No complications developed until hypertonic solutions of glucose were injected. The administration of solutions containing glucose in Experiment 2 resulted in some thrombosis of the vein in almost every rat and occlusion of the vein and leakage into the tissues in approximately 25 per cent of the animals. The results from animals in which leakage occurred were discarded. These complications occurred with equal frequency in rats with and without epinephrine. In Experiment 3, a small plastic tube was inserted in the jugular vein and directed towards the heart. This procedure did not cause occlusion and leakage at the point where the infusion fluid entered the vein from the catheter but there occurred respiratory distress and sharp decreases in the rate of work more frequently in the control animals than in animals which received epinephrine. In some instances thrombi were found occluding major vessels.

The animals were anesthetized with phenobarbital sodium and cyclopal sodium. Adrenal enucleations were performed by the procedure of Ingle and Nezamis (in press) and adrenalectomies by the procedure of Ingle and Griffith (1942). The animals were subjected to the work test immediately following the operation. Stimulation was continued until the muscle ceased to respond or for a maximum of 24 hours. Temperature was constant at 28±0.5°C.

## EXPERIMENTS AND RESULTS

One rat of each pair received 20 cc. of a solution containing epinephrine and the control animal received a solution without epinephrine.

In Experiment 1 (Table 1), adrenal enucleated rats were given ACE with and without epinephrine. Five groups of rats were given 4 cc. of ACE per 24 hours. The addition of epinephrine in amounts of 2, 5, 10, and 20 parts per million did not significantly improve work performance and 40 parts per million suppressed work performance. At this dose of ACE, 61 of 66 rats which received epinephrine died before the end of 24 hours and 53 of the 66 rats without epinephrine died.

TABLE 1. THE WORK PERFORMANCE OF ADRENAL ENUCLEATED RATS GIVEN ADRENAL CORTEX EXTRACT WITH AND WITHOUT EPINEPHRINE

| ACE cc. 24 hrs. | Ephinephrine concentration | Number of pairs | Total recorder revolutions. Averages and range | |
|---|---|---|---|---|
| | | | Epinephrine | Controls |
| 4 | 1:500,000 | 12 | 11507 ( 5584–16174) | 13641 ( 6796–35235) |
| 4 | 1:200,000 | 12 | 11682 ( 6880–16229) | 10813 ( 6678–19635) |
| 4 | 1:100,000 | 12 | 13825 ( 5593–28129) | 12622 ( 6092–23527) |
| 4 | 1: 50,000 | 18 | 10526 ( 5295–18797) | 13273 ( 5047–25994) |
| 4 | 1: 25,000 | 12 | 9566 ( 4858–15808) | 14239 ( 4011–24555) |
| 8 | 1:500,000 | 12 | 20119 (12242–28138) | 18222 ( 8047–22836) |
| 8 | 1:100,000 | 12 | 20514 ( 7007–30028) | 22518 (16780–27286) |
| 8 | 1: 50,000 | 12 | 9880 ( 6198–16295) | 18393 ( 6727–35638) |
| 15 | 1:500,000 | 12 | 21804 ( 7704–37764) | 21354 (11275–32303) |
| 15 | 1:100,000 | 12 | 17313 ( 7815–30526) | 24769 ( 8620–36004) |
| 15 | 1: 50,000 | 12 | 20154 ( 5506–37917) | 21753 ( 5756–38800) |

Three groups of rats were given 8 cc. of ACE per 24 hours. The addition of 2 and 10 parts of epinephrine per million did not significantly change work performance but 20 parts per million definitely suppressed work. At this dose of ACE, 22 of 36 rats which received epinephrine died during 24 hours and 8 of 36 rats without epinephrine died.

Three groups of rats were given 15 cc. of ACE per 24 hours. The addition of 2, 10, and 20 parts per million of epinephrine failed to improve work performance. At this dose of ACE, 24 of 39 rats which received epinephrine died during 24 hours and 10 of 39 rats without epinephrine died.

In Experiment 2 (Table 2), all of the rats received a glucose load of 200 mgm. per 100 grams of rat per hour. Twelve pairs of adrenal enucleated rats were given 8 cc. of ACE per 24 hours. The addition of 2 parts per million of epinephrine did not improve work performance.

TABLE 2. THE WORK PERFORMANCE OF ADRENALLY INSUFFICIENT RATS GIVEN ADRENAL CORTEX EXTRACT AND GLUCOSE WITH AND WITHOUT EPINEPHRINE. GLUCOSE LOAD OF 200/100/h

| ACE cc. 24 hrs. | Epinephrine concentration | Operation | Number of pairs | Total recorder revolutions. Averages and range | |
|---|---|---|---|---|---|
| | | | | Epinephrine | Controls |
| 8 | 1:500,000 | enucleation | 12 | 23177 (7091 –47556) | 24843 (11028–42299) |
| 12 | 1:500:000 | enucleation | 15 | 38702 (14521–53909) | 39821 (26467–53753) |
| 15 | 1:50,000 | enucleation | 6 | 20307 (6458–50342) | 42002 (33493–53535) |
| 15 | 1:500,000 | adrenalectomy | 19 | 45830 (30940–59031) | 45946 (37760–60140) |

Five of the 12 rats which received epinephrine died during 24 hours and 6 of the 12 rats without epinephrine died.

Fifteen pairs of adrenal enucleated rats received glucose with 12 cc. of ACE per 24 hours. The addition of 2 parts per million of epinephrine did not improve work performance. Two of the 15 rats which received epinephrine died during 24 hours and 1 of the 15 rats without epinephrine died.

Six pairs of adrenal enucleated rats received glucose with 15 cc. of ACE per 24 hours. The addition of 20 parts of epinephrine per million suppressed work. Four of the 6 rats which received epinephrine died during 24 hours and none of the rats without epinephrine died.

Nineteen pairs of surgically adrenalectomized rats received glucose with 15 cc. of ACE per 24 hours. The addition of 2 parts of epinephrine per million did not improve work. None of the rats died in this series.

TABLE 3. THE WORK PERFORMANCE OF ADRENALLY INSUFFICIENT RATS GIVEN ADRENAL CORTEX EXTRACT AND GLUCOSE WITH AND WITHOUT EPINEPHRINE. GLUCOSE LOAD OF 200/100/h. INFUSED THROUGH PLASTIC TUBE TIED INTO THE JUGULAR VEIN AND DIRECTED TOWARDS THE HEART

| ACE cc. 24 hrs. | Epinephrine concentration | Operation | Number of pairs | Total recorder revolutions. Averages and range | |
|---|---|---|---|---|---|
| | | | | Epinephrine | Controls |
| 15 | 1:500,000 | enucleation | 9 | 42038 (15744–53960) | 38584 (26162–49940) |
| 15 | 1:100,000 | enucleation | 12 | 39949 (7793 –55458) | 37380 (13520–55666) |
| 15 | 1:500,000 | adrenalectomy | 20 | 43030 (25130–57325) | 36927 (15111–52975) |
| Total averages with standard deviation of average | | | | 41910 (1731) | 37688 (1484) |

Difference in averages with standard deviation of the difference—4220 (2280)—Ratio—1.85

In Experiment 3 (Table 3), the infusions were made through a plastic tube which was tied into the jugular vein and directed towards the heart. Nine pairs of adrenal enucleated rats were given glucose with 15 cc. of ACE per 24 hours. The animals which received 2 parts per million of epinephrine performed a greater average amount of work than did the animals without epinephrine. Similar results were obtained with 12 pairs of adrenal enucleated rats given glucose and 15 cc. of ACE per 24 hours when 10 parts per million of epinephrine were added and in 20 pairs of surgically adrenalectomized rats given glucose and 15 cc. of ACE per 24 hours when 2 parts per million of epinephrine were added. Three of the 41 rats given epinephrine in this experiment died during 24 hours and 6 of the rats without epinephrine died. It was noted that a number of the rats in this experiment exhibited respiratory distress and sharp decreases in work output associated with a thrombus in the vessels of the heart or lungs. These complications occurred more frequently among the animals which did not receive epinephrine. When the results for all of the animals which received epinephrine in Experiment 3 were grouped together, the average was 41,910 recorder revolutions with a standard devia-

tion of the average of 1,731. The average for the control animals was 37,688 recorder revolutions with a standard deviation of the average of 1,484. The ratio between the difference (4,220) and the standard deviation of the difference (2,280) was 1.85. From the statistical standpoint the probability is 93 chances in 100 that the performance of the rats treated with epinephrine in Experiment 3 was better than the performance of the controls. It is concluded, however, that the group difference was probably based upon the lower frequency of circulatory complications in the epinephrine-treated rats and therefore represents an artifact of methodology rather than any direct benefit of epinephrine upon the ability of the adrenally insufficient rat to sustain work output.

## DISCUSSION

Although epinephrine may cause a temporary increase in the work output of the adrenally insufficient rat, just as it does in fatigued normal animals, it fails to increase the total energy output of the animal when given alone or in combination with ACE. Large doses of epinephrine caused a decrease in work. Adrenal demedullated rats show normal voluntary activity (Ingle and Harris, 1936) and a normal total work output when subjected to stimulation of the gastrocnemius muscle (Ingle, Hales and Haslerud, 1936) although it was observed (Ingle and Lukens, 1941) that the adrenal demedullated rat shows a lower than normal rate of work during the first few hours of muscle stimulation. It is not clear as to whether this difference was due to the absence of the adrenal medulla or to a decrease in the secretory activity of the regenerated cortex of the demedullated gland. The physiological role of the adrenal medulla has remained difficult to clarify because any means of inactivating this portion of the gland may possibly limit the secretory activity of the cortex. All of the data from this and previous studies by one of us (D.J.I.) are consistent with the concept that if the adrenal medulla plays a role in body economy, it is manifest principally in acute emergencies rather than by sustaining resistance to prolonged stress.

The data from surgically adrenalectomized animals were in agreement with the results from animals having the adrenal glands inactivated by enucleation without surgery. There was some evidence (Experiment 3, Table 3) that epinephrine reduced the number of complications resulting from the intravenous administration of hypertonic solutions of glucose. The group differences in work (Table 3), if reliable, would seem to represent an example of an artifact in results rather than a true beneficial effect of epinephrine upon work.

## SUMMARY

Surgically adrenalectomized rats and adrenal enucleated rats were subjected to faradic stimulation of both hind legs five times per

second until death or for 24 hours. Each rat (200 grams body-weight) received continuous intravenous infusions at the rate of 20 cc. per 24 hours. The solution contained 0.9 per cent sodium chloride, 0.1 per cent ascorbic acid, various concentrations of adrenal cortex extract (ACE), and epinephrine was added to the solution given one animal of each pair. Glucose was added in some experiments. The total work output was proportional to the amount of ACE administered but the addition of epinephrine was without apparent direct effect upon total work. Epinephrine in amounts of 20 and 40 parts per million suppressed work. Work was greatly improved by the addition of 200 mgm. of glucose per 100 grams of rat per hour.

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# A NEW REAGENT FOR THE HISTOCHEMICAL DEMONSTRATION OF ACTIVE CARBONYL GROUPS. A NEW METHOD FOR STAINING KETONIC STEROIDS[1]

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PRESENT methods for demonstrating the carbonyl groups of ketosteroid are based on their reaction with phenylhydrazine (Bennett, (1940) or with 2, 4-dinitrophenyl hydrazine (Dempsey and Wislocki, 1946; and Albert and Leblond, 1946) to form colored hydrazones (yellow or orange). Further evidence that these fat-soluble, carbonyl-containing substances were indeed ketosteroids was provided by localization of other steroid reactions and properties in the same areas as the positive phenylhydrazine reactions. These were fluorescence, birefringence, anisotropism after reaction with digitonin, Liebermann-Burchardt test, Schiff reaction, reduction of alkaline silver solutions, and sudanophilia (Dempsey, 1946). Still other evidence was afforded by correlation of the steroid reactions with different physiological states (Dempsey and Wislocki, 1946; Albert and Leblond, 1946; Greep and Deane, 1947; Deane and Shaw, 1947; Deane and Greep, 1947; and Wislocki, 1949).

By the application of these techniques, ketosteroid has been demonstrated in the outer portion of the zona fasciculata of the adrenal cortex in cats and dogs, and in the zona glomerulosa as well in rats and humans (Bennett, 1940; Dempsey and Wislocki, 1944; Deane and Greep, 1946; and Dempsey and Wislocki, 1946), in the interstitial cells of the mammalian testis (Pollack, 1942), in the cells of the theca interna of the follicle and the luteal cells of the rat's ovary (Dempsey and Bassett, 1943) and in the syncytial trophoblast of the human placenta (Wislocki and Bennett, 1943).

In order to improve the sensitivity of the phenylhydrazine reac-

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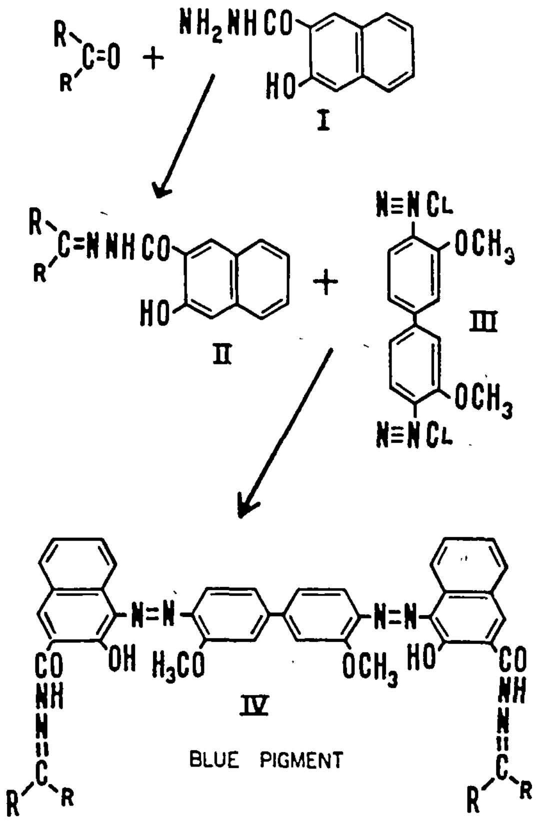
[2] On leave of absence from the Hebrew University, Jerusalem, Israel. Research fellow in surgery, Harvard Medical School. This fellowship was made possible by a grant from the Jane Coffin Childs Memorial Fund for Medical Research, Yale University.

tion by increasing the color value of the final compound, reagents were synthesized which contain a hydrazine group and which react with active carbonyl groups to form nearly colorless substances, but which may be converted by coupling with a tetrazonium compound into an intensely blue azo dye (Seligman and Ashbel, 1949). The best reagent for this purpose proved to be the hydrazide of 2-hydroxy-3-naphthoic acid (I) (Seligman, Friedman, and Herz, 1949). This was found to react *in vitro* with 3, 17, and 20-ketosteroids, but, as was expected, not to react with 11-ketosteroid. Frozen sections, prepared from formalin-fixed tissue, were washed free of formalin and reacted with the hydrazide (I) in alcohol-water-acetic acid solution. The reaction product (II) was insoluble in alcohol-water, so that the excess reagent was readily removed by washing. The frozen sections were then treated in 50 per cent alcohol at pH 7.2 with tetrazotized diorthoanisidine (III).[3] The development of a blue pigment (IV) in the areas which contained the lipoid with active carbonyl groups was rapid.




[3] This material is available commercially under the name of Dupont Naphthanil Diazo Blue B in stable, powder form and contains 20% tetrazotized diorothoanisidine, 5% zinc chloride, and 20% aluminum sulfate. It was provided through the courtesy of Dr. E. R. Laughlin, Dupont de Nemours and Co., Boston, Mass.

## METHOD

Fresh tissue was fixed in 10 per cent formalin, neutralized by standing over calcium carbonate or with phosphate buffer (Lillie, 1948). The period of fixation was varied from several hours to several weeks, and in a few experiments for several months, without significant change in the final picture. In a few instances formalin was perfused through the tissue by intracardiac injection. After fixation the formalin was removed by washing the blocks in running tap water for 24 hours, or frozen sections (15–30 microns in thickness) were prepared and were washed in several changes of water for a few hours or longer.

The washed frozen sections were then placed at room temperature for 1–3 hours in an 0.1 per cent solution of 2-hydroxy-3-naphthoic acid hydrazide (I). This solution was prepared by dissolving the finely powdered hydrazide in 50 per cent ethyl alcohol (prepared from aldehyde-free absolute ethanol) containing 5 per cent glacial acetic acid, with the aid of warming (60–70°C.) and shaking. This solution could be stored at room temperature for periods as long as 12 days without alteration. The period of reaction with the tissue could be shortened to 30 minutes by incubation at 50°–60°C. The sections were then washed in several changes of 50 per cent alcohol for 2 hours followed by several changes of water for 2 hours.

The sections were then placed in a solution prepared from equal volumes of absolute alcohol and 1/15M phosphate or acetate buffer, pH 7.2–7.5. To 50 cc. of this solution containing the sections at room temperature, 50 mgm. of the stabilized tetrazonium powder (III) was added and stirred-in or a very fresh solution of the powder in a few cc. of cold water was added and stirred. The development of a blue color in the tissue reached a maximum of intensity in 0.5–2 minutes. The yellow solution also darkened during this period. The sections were transferred with a glass rod to water and washed with a few changes of water for 5–15 minutes. The sections were then mounted on glass slides with Kaiser's glycerogel. To avoid bubbles the sections were covered with glycerogel and were placed in the incubator for a few minutes before the cover slip was applied. The azo dye has not changed in the sections in over one year.

In general the shorter periods of incubation and of washing were used with the thinner sections. The periods of washing given above are considerably longer than was actually necessary by a safe margin. It was found that the addition of the tetrazonium compound to the sections in buffer-alcohol was a critical step in the procedure, because when the sections were added to the buffer-alcohol in which the tetrazonium compound had been dissolved previously, the development of color was poor. The concentration of alcohol was also important.[4] This may have been due in part to the rapid decomposition of the tetrazonium compound which occurs in aqueous solutions and to the necessity of coupling in tissue sections in a two-phase system; i.e.

[4] While the manuscript of this paper was being prepared, a note appeared in *Nature* (Camber, 1949) describing the use of 2-hydroxy-3-naphthoic acid hydrazide for staining ketosteroid in the adrenal cortex with diazotized amines. The procedure outlined is unlike the procedure herein described, and corresponds closely to the procedure (conducted in aqueous solution) which we first used in October, 1947, but with which we were not satisfied, because the degree of staining was variable and weak as compared to results obtained with the method eventually evolved.

the steroids and their reaction product (II) formed with the hydrazide is dissolved in the fat droplets, and the tetrazonium salt in aqueous solution is poorly and slowly diffusible into lipoid. At higher pH than that recommended, decomposition of the tetrazonium salt proceeds at a more rapid rate and results in a yellowish-brown cast to the sections. At lower temperatures the decomposition of the tetrazonium compound is slower, but the reaction rate is also slower. This slowing of the reaction rate is not due to a slower rate of coupling with the napththol, which proceeds rapidly in aqueous media even at 4°C., but must be due to a decrease in the solubility in lipoid of the steroid-hydrazide reaction product (II).

## RESULTS WITH TISSUES WHICH CONTAIN KETOSTEROID

### *Adrenal Gland*

The adrenal glands from 15 normal *rats* (killed with ether) were stained by the present method for carbonyl groups (figs. 7 and 8). Blue color was produced in the zona glomerulosa (11 were intense, 3 were weak and 1 did not stain). Blue color in the zona fasciculata was seen in 14 and 1 did not stain. The zona reticularis stained in 7, weakly in 1 and not at all in 8. The lipoid droplets of the cells were stained, and in addition the dye occurred in diffuse, granular-appearing deposits in the cytoplasm. The nuclei were not stained (fig. 8). The adrenal medulla stained a slight pink color in some of the glands. In no instance was any blue color seen in this location. The period of incubation with the hydrazide varied from 20 minutes to 7 hours. Most of the sections were incubated for 1–3 hours. No correlation of time and degree of staining was noted within these limits.

The adrenal glands from 13 ether-killed *rabbits* which had received urine for the Aschheim-Zondek test for pregnancy (most were negative) were stained by the present method for carbonyl groups.

The zona glomerulosa was intensely blue in 10 and weak in 3. The zona fasciculata was intensely blue in all 13. The zona reticularis was stained in 4, weakly in 3, and not at all in 6. In some sections the

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PLATE I

FIG. 1. Dog adrenal. The fasciculata contains most of the lipoidal carbonyl groups. The glomerulosa (left) is paler than noted in other species. The reticularis contains scattered cells which were stained, unlike most of the specimens examined. ×50.

FIG. 2. Rat testis. Interstitial cells which contain blue-stained fat droplets in their cytoplasm. The nuclei are unstained. ×1500.

FIG. 3. Rat cerebellum. The white matter is deeply stained and the molecular layer of the cortex is delicately stained. The granular layer of the cortex contains blue-stained extensions of nerve fibers from the white matter. The blue-stained material was not extractable with lipoid solvents. ×15.

FIG. 4. Dog adrenal. All layers of the cortex stain blue for lipoidal carbonyl groups. The capsule (top) and the medulla (bottom) are stained pink (not indicative of carbonyl groups). × 50.

FIG. 5. Dog ovary. Corpus luteum (upper right) is stained intensely blue for lipoidal carbonyl groups. Follicles contained a variable amount of delicate blue in the granulosal cells. ×50.

FIG. 6. Rat placenta (near term). The syncytial cells contain blue-stained fat droplets. ×900.

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG.

fusely dispersed dye. The nuclei were unstained. Cells of the theca interna in the outer portions of the corpora lutea were more intensely stained than the lutein cells in the center, as were also special stellate cells scattered throughout the corpus luteum, described for human ovaries by White, Hertig, Rock, and Adams, 1949. Cells of the interstitial glands of the ovary contained blue fat droplets. Theca folliculi also was stained blue. In addition the lining cells of the sinusoids of the corpora lutea were stained blue. The capillary stain in contrast to other areas was not removed by lipoid solvents, nor was it prevented by preparing paraffin embedded sections. The inner elastic layers of some blood vessels were stained blue.

The ovaries of 2 normal *dogs* were examined by this method. The corpora lutea were stained intensely blue (fig. 5). The cytoplasm of the lutein cells contained many blue fat droplets of various sizes. Young follicles exhibited a delicate violet pattern in the cytoplasm of the ova (fig. 12), and the theca folliculi cells contained bluish-violet staining nuclei (fig. 14). The stain in these cells was not altered by extraction with lipoid solvents. The older follicles were darker blue, due presumably to staining of the granulosal cells (fig. 13). The internal elastic layer of blood vessels of the ovary were stained blue.

One dog was 6 weeks post-partum. The large corpora lutea were intensely blue. The dense fibrous septa were violet-pink, and the fine reticulum of the intercellular boundaries were blue. The lutein cells were large and vacuolated (figs. 16 and 17). They contained blue lipoid droplets and blue finely dispersed dye. This was especially noticeable in the theca cells of the periphery of the corpus luteum. In some sections the theca folliculi contained cells whose nuclei stained purple. They stain was not removed by lipoid solvents. These cells were not easily distinguished from the remaining ovarian stroma in eosin-hematoxylin sections.

Corpora lutea of *humans* stained less intensely than in the dog. The stain was finely dispersed in the cytoplasm of the thecal lutein cells (fig. 15, 24 days after menstruation). The nuclei were not colored. Darkly stained stellate cells, similar to those described by White *et al.* were scattered throughout the corpus luteum in a radiating pattern. Cells of the theca externa also stained blue, but this blue dye was not readily removed by lipoid solvents. The interstitial cells of a corpus atreticum were stained blue, but most of the blue colored material was not lipoid soluble.

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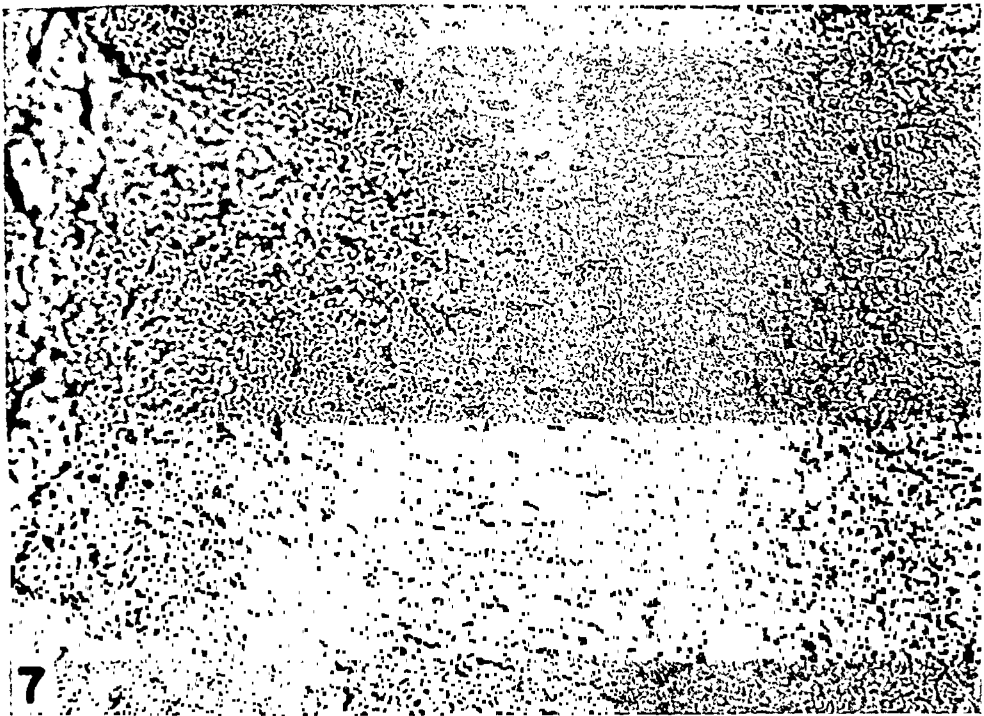
PLATE II

FIG. 7. Rat adrenal. The reaction for lipoidal carbonyl groups is evident in the glomerulosa (right) and part of the fasciculata. Sinusoids in the fasciculata, reticularis and medulla were also stained, but this colored material was not extractable with lipoid solvents. Photographed through a red filter. × 180.

FIG. 8. Same section as Fig. 7, showing more stain in the glomerulosa (right) than the fasciculata. The nuclei are outlined by the stained fat droplets and finely dispersed dye in the cytoplasm. Photographed through a red filter. ×1200.



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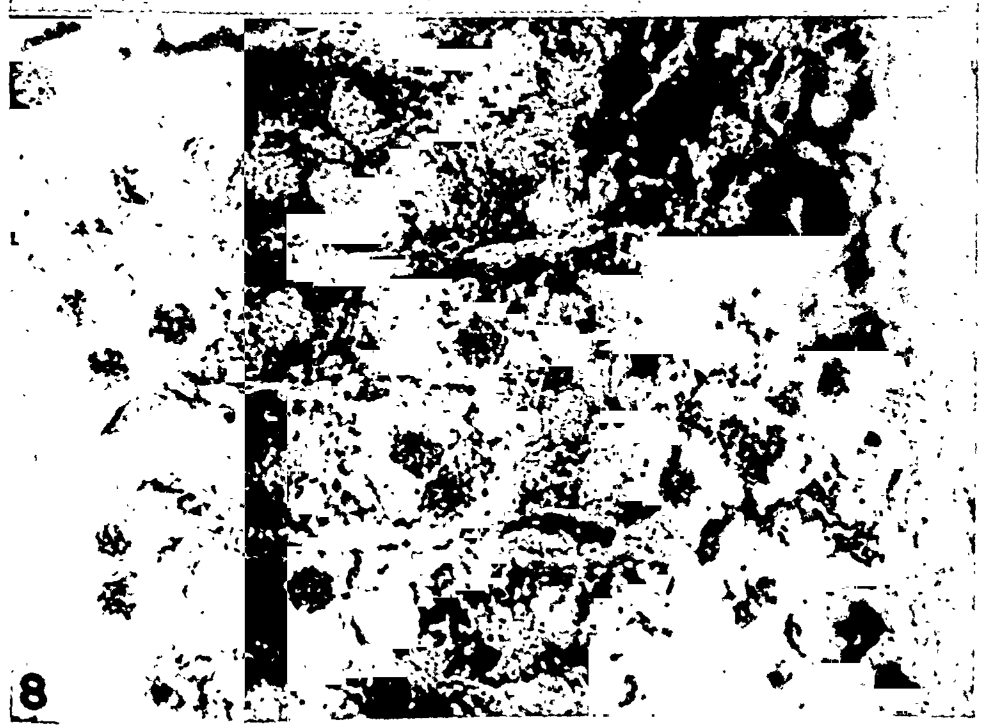

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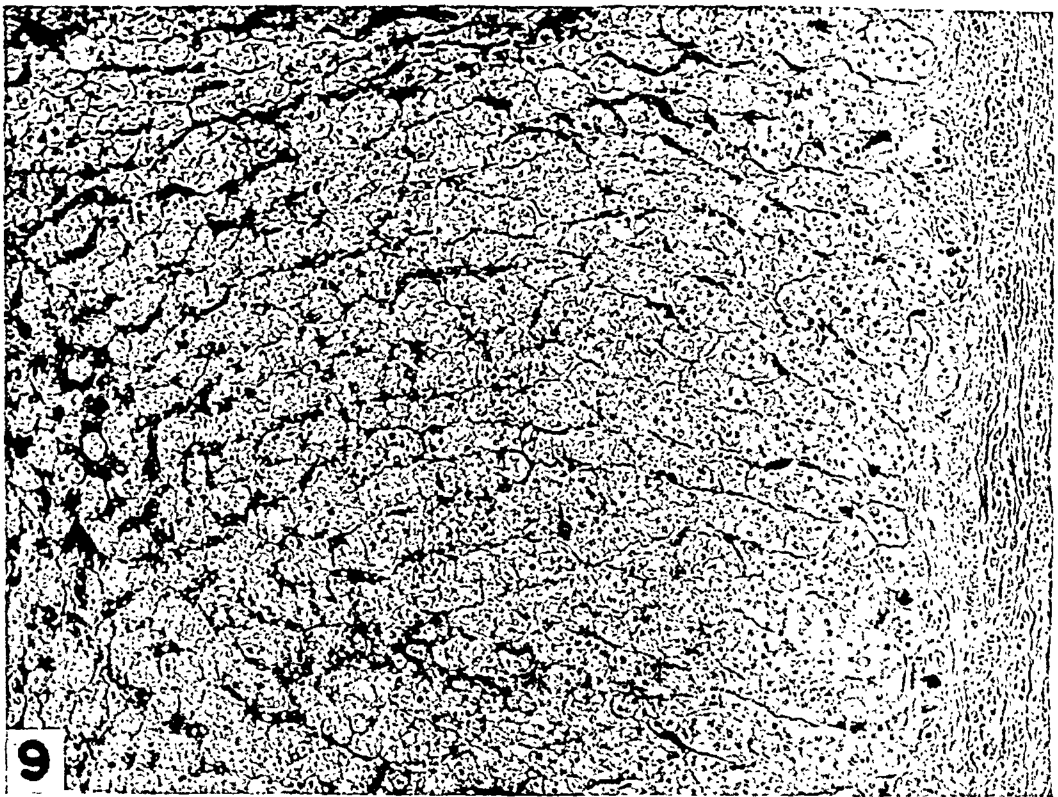

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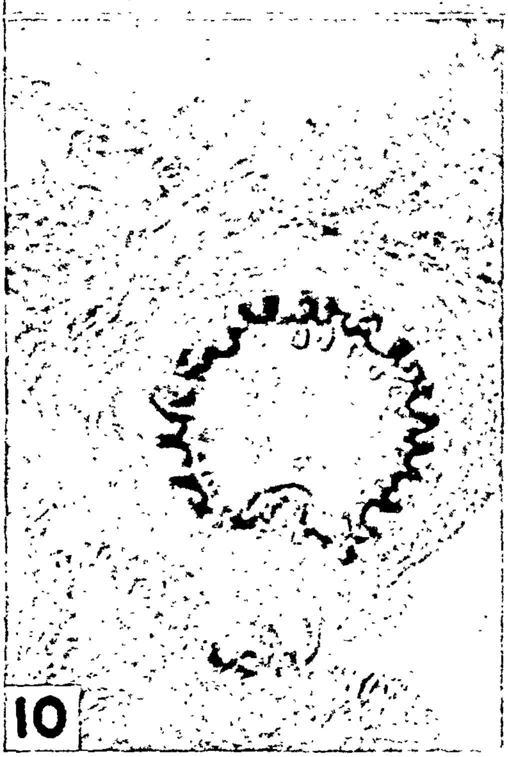

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*Placenta*

The placenta of a *rat* near term gave an intense blue color in lipoid droplets in the syncytial throphoblast of the placental labyrinth (figs. 6 and 18). In some areas the cytoplasm was stained diffusely blue, although not sufficiently to mask the droplets. The droplets varied in size and were located predominantly on the fetal side of the syncytium (fig. 18). In addition there was a positive reaction of the cytoplasm of the trophoblastic giant cells located around the margin of the placental disk.

The placentas of a *rat* and 6 *mice* from mid-pregnancy stained similarly but weak'y. Of three *human* placentas at term, only one showed isolated, scattered, blue-staining droplets in the flattened syncytial trophoblasts.

*Testis*

The testis of 1 *rat*, 1 *rabbit*, and 3 *dogs* were stained for lipoidal carbonyl groups. Fat droplets in the cytoplasm of the interstitial cells stained blue. The nucleus was unstained and was outlined by the surrounding blue droplets (fig. 2).

*Other tissues*

Lipoidal carbonyl groups were not demonstrable by this method in lung, liver, kidney, spleen, pancreas, skeletal muscle, subcutaneous or mesenteric fat, intestine, uterus, prostate, epididymis, seminal vesicles, thyroid, pituitary, or blood smears.

## REACTION WITH NON-LIPOID MATERIAL IN TISSUES

*Fibrous tissues.*

In some of the sections, particularly of human adrenal tissue, a pink color was noted in fibrous tissue (fig. 4). This could not be prevented by prolonged washing. It was probably due to attachment of traces of the hydrazide (I) to protein or collagen through the naphtholic group. Coupling with the tetrazonium compound under these circumstances would occur into the free hydrazino group of the hydrazide. In *in vitro* experiments, it was found that the tetrazonium compound gave a red color with the hydrazide (I). However, when the hydrazino-group was tied-up by reaction with acetone or for-

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PLATE III

FIG. 9. Human adrenal cortex. Formalin-fixed tissue was carried through the paraffin embedding procedure, which removed lipoidal carbonyl-containing material. Only intercellular reticulum and sinusoids were stained blue. Photographed through a red filter. ×180.

FIG. 10. Dog arteriole in neurovascular bundle. Alcohol fixed. The inner elastic membrane is stained intensely blue. ×1200.

FIG. 11. Dog sciatic nerve. Single nerve fiber showing chicken-wire pattern in myelin sheath. The blue colored material in this network was insoluble in lipoid vents. ×2200.

maldehyde, coupling could only occur ortho to the hydroxy group. This type of coupling resulted in the production of a blue color. Treatment of stained sections with lipoid solvents such as absolute alcohol or acetone which slowly removed the blue compound, did not affect the non-specific pink color seen in fibrous tissue. This was considered evidence of attachment of the hydrazide (I) to protein in areas stained pink, and of attachment to lipoid carbonyl groups in areas stained blue. Further evidence was provided that the naphtholic group was important in attachment to tissue for the development of the pink color, by the preparation of 2-naphthoic acid hydrazide (Seligman, Friedman and Herz, 1949). When this material was used instead of the hydrazide (I), the sections were complete blanks (neither blue for ketosteroid nor pink in fibrous tissue). However, after blocking the carbonyl groups with this reagent, subsequent treatment with the hydrazide (I) led to development of the pink color in fibrous tissue, even though the blue color of carbonyl groups was permanently blocked. Other blocking agents such as hydrazine hydrate and hydroxylamine permanently blocked both the pink and blue colors after subsequent treatment with the hydrazide (I). This was probably due to the strong basic character of these agents.

*Nervous tissue*

In some sections of adrenal gland which included segments of nerve, a brilliant blue color was produced by the reaction in the myelin sheaths. This pigment, however, was not removed by the lipoid solvents which successfully removed the blue stain produced by carbonyl groups in lipoid. Subsequent investigation revealed that this reaction occurred regularly in formalin-fixed white matter of central (fig. 3) and peripheral nervous tissue (fig. 11). The active carbonyl groups involved in the reaction with nervous tissue were found only after formalin fixation, or in alcohol-fixed tissue subsequently exposed to formalin. A weak reaction was also observed with alcohol-fixed tissue subsequently treated with mineral acid or mercuric chloride. Unlike the reaction with lipoid, the reaction persisted in formalin-fixed tissue which was carried through the paraffin embedding process. In all cases, the reaction was blocked by prior treatment with hydrazine hydrate. Fractionation of fresh white matter of brain revealed that the reaction occurred in the protein fraction only after subsequent exposure to formalin. This explains the discrepancy between the amount of aldehyde demonstrated in brain by the Schiff reagent and the small amount of aldehyde actually isolated

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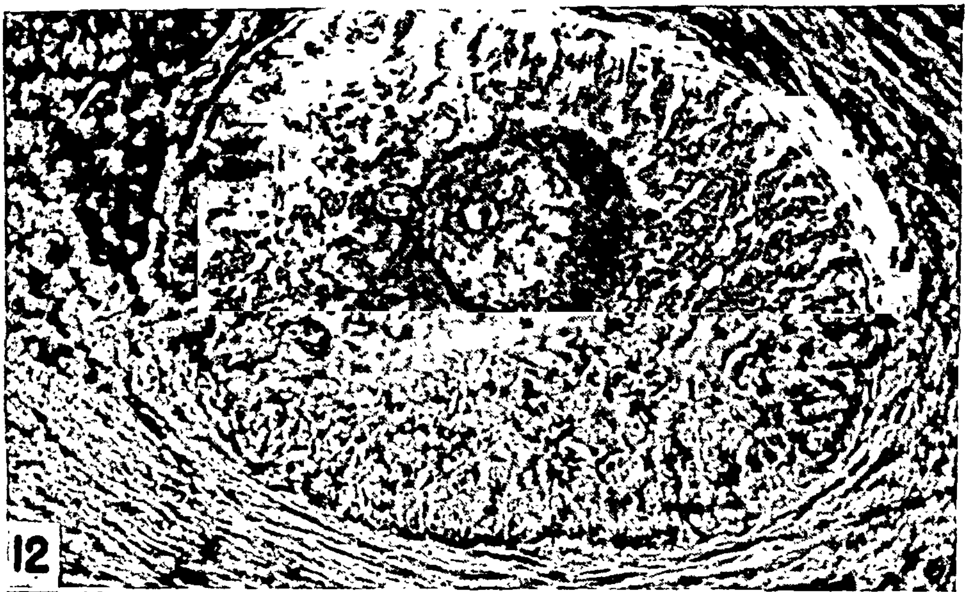
PLATE IV

FIG. 12. Dog ovary. A young follicle was stained a delicate bluish-violet in the cytoplasm of the ovum. The granulosa cells were not stained blue. ×800.

FIG. 13. Dog ovary. An older follicle stained intensely blue, presumably in the enveloping granulosa cells. ×800.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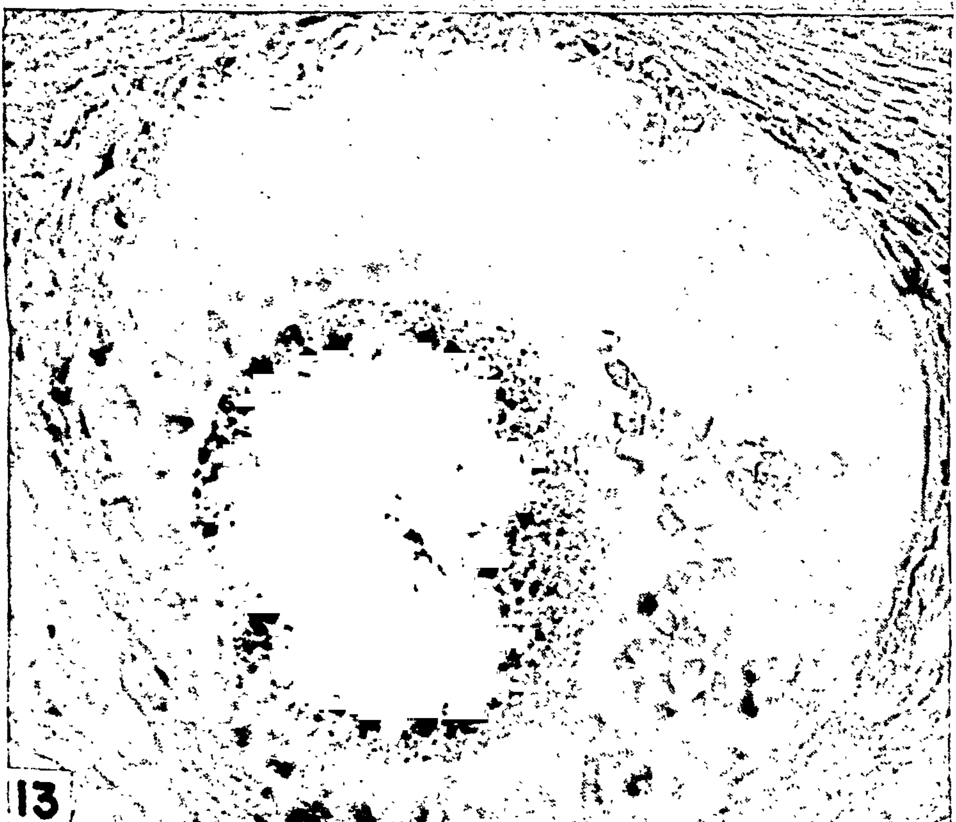
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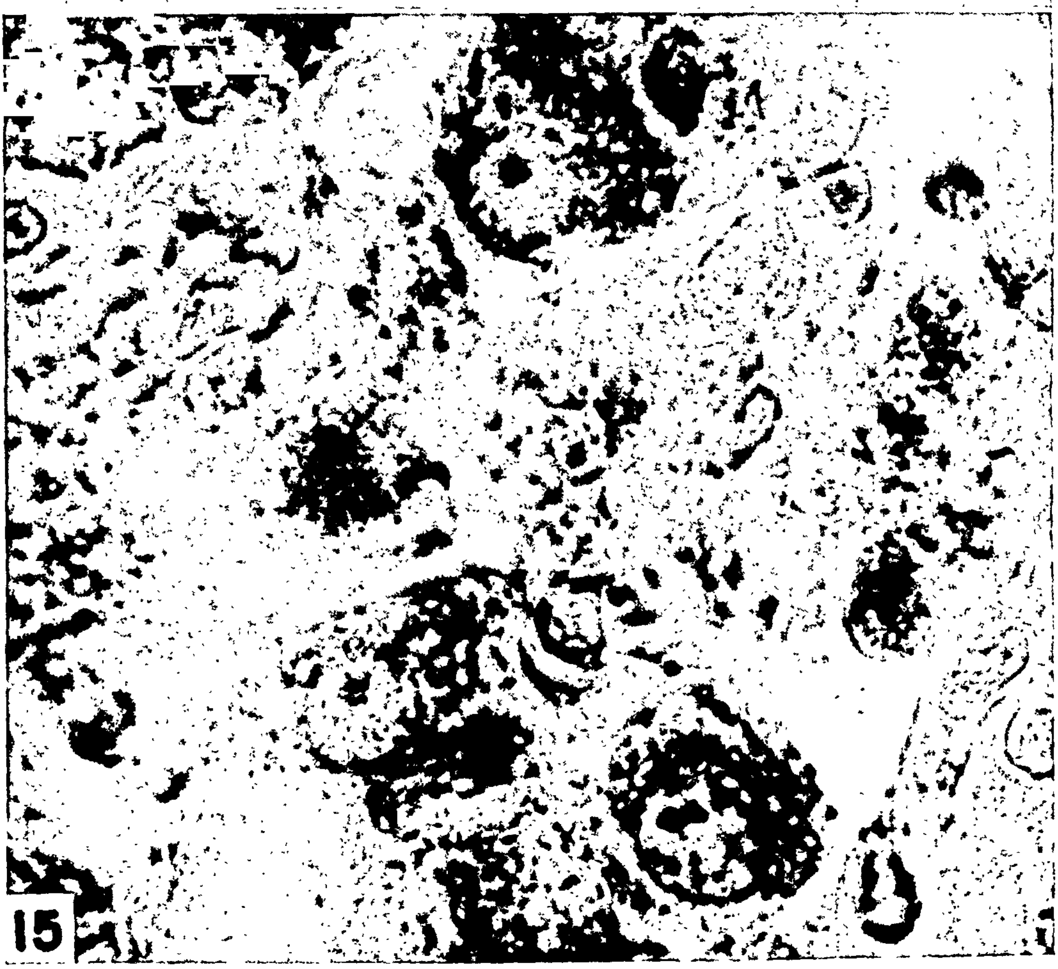
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from brain (Anchel and Waelsch, 1942). Further study of the results with nervous tissue will form the subject of another communication (Seligman and Ashbel, 1949).

*Elastic tissue*

The wavy elastic layer of large blood vessels was found to react with the hydrazide to give an intense blue stain (fig. 10). This stain was not removed by lipoid solvents and its production was blocked by prior treatment with hydrazine hydrate. However, the reaction differed from that of the adrenal cortex and nervous tissue, in that exposure to formalin was not required to unmask the carbonyl groups. This was demonstrated by successful staining of alcohol-fixed tissue. Similar staining of elastic tissue has been observed with other methods for demonstrating carbonyl groups (Feulgen and Voit, 1924; Albert and Leblond, 1946; Dempsey, 1946; and Shreck and McNamara, 1947).

*Sinusoidal reticulum*

After extraction with acetone of all stained lipoid in adrenal cortex, blue color remained in the lining membranes of many of the sinusoids (fig. 7). This was also observed in formalin-fixed tissue which had been carried through the paraffin embedding process (fig. 9).

## IN VITRO REACTIONS WITH PURE MONOKETOSTEROIDS

In order to compare the relative reactivity of the 3, 11, 17 and 20 keto groups, which occur singly or in combination in various ketosteroids, compounds[6] were selected which contained only one of these keto groups in each. Thus, a 3-keto-group was tested in testosterone, an 11-keto-group in the methyl ester of 3-hydroxy-11-keto aetio cholonic acid, a 17-keto group in oestrone, and a 20-keto group in pregnen-β-3-ol-one-20. Estradiol and recrystallized cholesterol served as non-ketonic steroids.

Each steroid (5 mg.) was dissolved in 10 cc. of hot absolute ethanol and the solution was cooled to room temperature. A solution of the hydrazide (I) (10 mg.) in absolute ethanol (10 cc.) or in 50% alcohol

[6] These compounds in crystalline form were provided us through the courtesy of Professor Louis F. Fieser, Harvard University.

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PLATE V

FIG. 14. Dog ovary (6 weeks post-partum). Theca folliculi cells stained purple in the nuclei. This colored material was not lipoid soluble. Photographed through a red filter. ×150.

FIG. 15. Human corpus luteum (24 days post-menstrual). Theca interna cells in the periphery of the corpus luteum contain fine, blue-stained, lipoid droplets in the cytoplasm. Nuclei were not stained. The nucleoli were not colored blue. Photographed through a red filter. ×2000.

was added, followed by 1 drop of glacial acetic acid. In some experiments the solution was heated to boiling and then cooled; in others, the reaction proceeded at room temperature for various periods. The reaction was terminated by the addition of several drops of freshly distilled pyruvic acid, which converted the excess hydrazide to an alkali soluble hydrazone. After standing for an hour or after heating to boiling, 25 cc. of benzene and 25 cc. of ethyl acetate was added and the mixture was exhaustively extracted with dilute sodium bicarbonate solution (5%) until the washings failed to color on addition of

TABLE 1. EXTENT OF REACTION OF 2-HYDROXY-3-NAPHTHOIC ACID HYDRAZIDE WITH PURE STEROIDS UNDER VARIOUS CONDITIONS

| Compound | Position of the keto-group | Reaction in boiling benzene | Reaction in boiling alcohol for 1 minute and room temperature for 24 hours | Reaction in boiling alcohol for 1 minute | Reaction in alcohol at room temperature for one hour |
|---|---|---|---|---|---|
| Testosterone | 3 | +++++ | ++++ | +++ | 0 |
| 3-Hydroxy-11-keto aeteo cholanic acid methyl ester | 11 | 0 | 0 | 0 | 0 |
| Estrone | 17 | +++ | +++++ | +++ | +++ |
| Pregnen-β-3-ol-one-20 | 20 | + | +++ | ++ | 0 |
| Estradiol | — | 0 | 0 | 0 | 0 |
| Cholesterol | — | 0 | 0 | 0 | 0 |

the tetrazonium compound (III). The organic layer was then shaken with a solution of the tetrazonium compound containing a little sodium bicarbonate. The intensity of the blue-red dye in the organic layer indicated the extent of reaction of the hydrazide with the steroid. Table 1 gives the results of the reaction under various conditions. The most active carbonyl groups were 3 and 17. The 20-keto group was less reactive, and the 11-keto group did not react at all. The Schiff reagent did not react with any of these ketosteroids dissolved in dilute alcohol or dilute propylene glycol within a period of several hours, or with a solution of testosterone in tricaprylin (10 mg. per cc.).

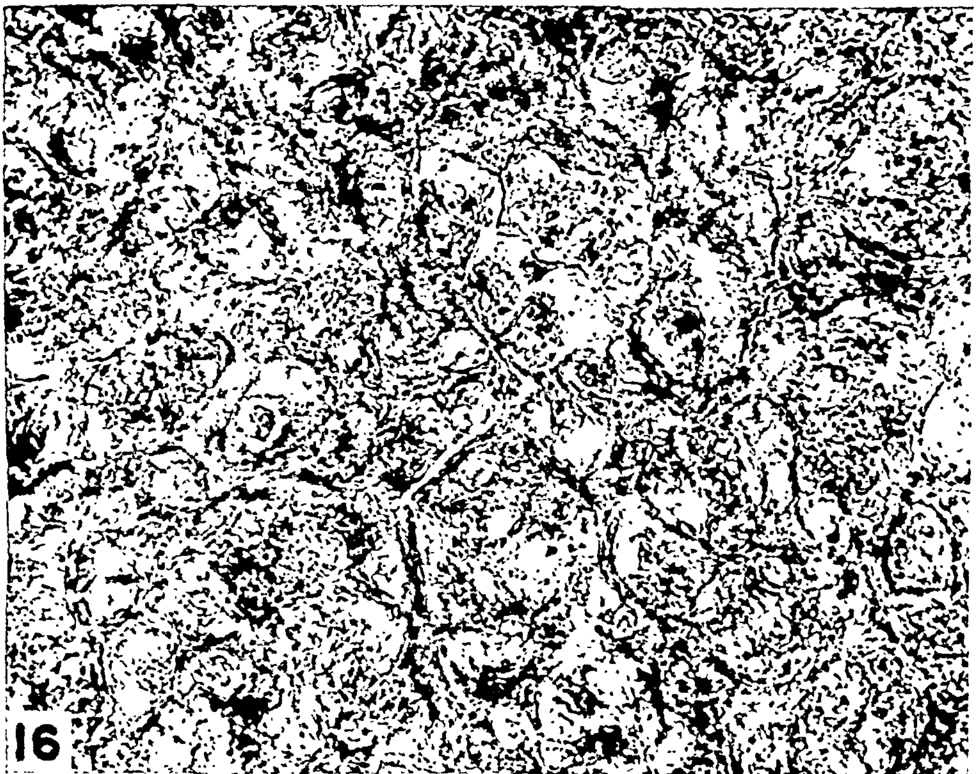
## SPECIFICITY OF THE REACTION

Doubt has been cast upon the specificity of the phenylhydrazine reactions for demonstrating ketosteroid in the lipoid of the various sites enumerated above by several authors (Gomori, 1942; Albert and Leblond, 1946; Oster and Oster, 1946; and others quoted in Dempsey, 1948), on the grounds that lipoidal aldehydes (stearyl and palmityl aldehydes) are readily liberated from plasmalogens by hydrolysis

PLATE VI

FIG. 16. Dog ovary (6 weeks post-partum). The lutein cells are vacuolated and contain a fine network of blue-stained fat droplets and granules at the cell periphery. Lipoid solvent extraction removed the blue material. Photographed through a red filter. ×800.

FIG. 17. Same section as Fig. 16. Eosin-hematoxylin stain. Cell nuclei and vacuolization are shown. ×800.



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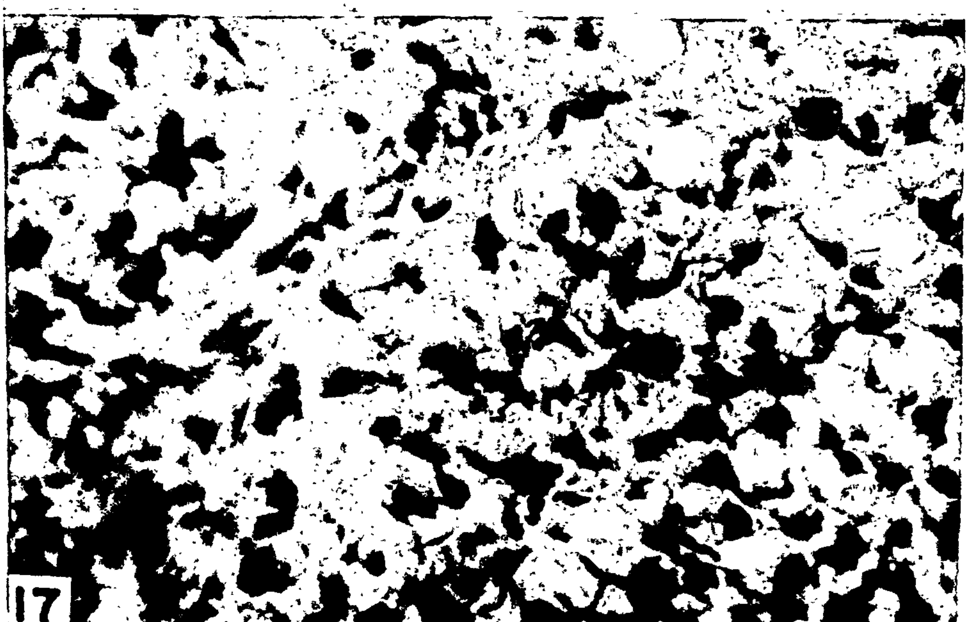

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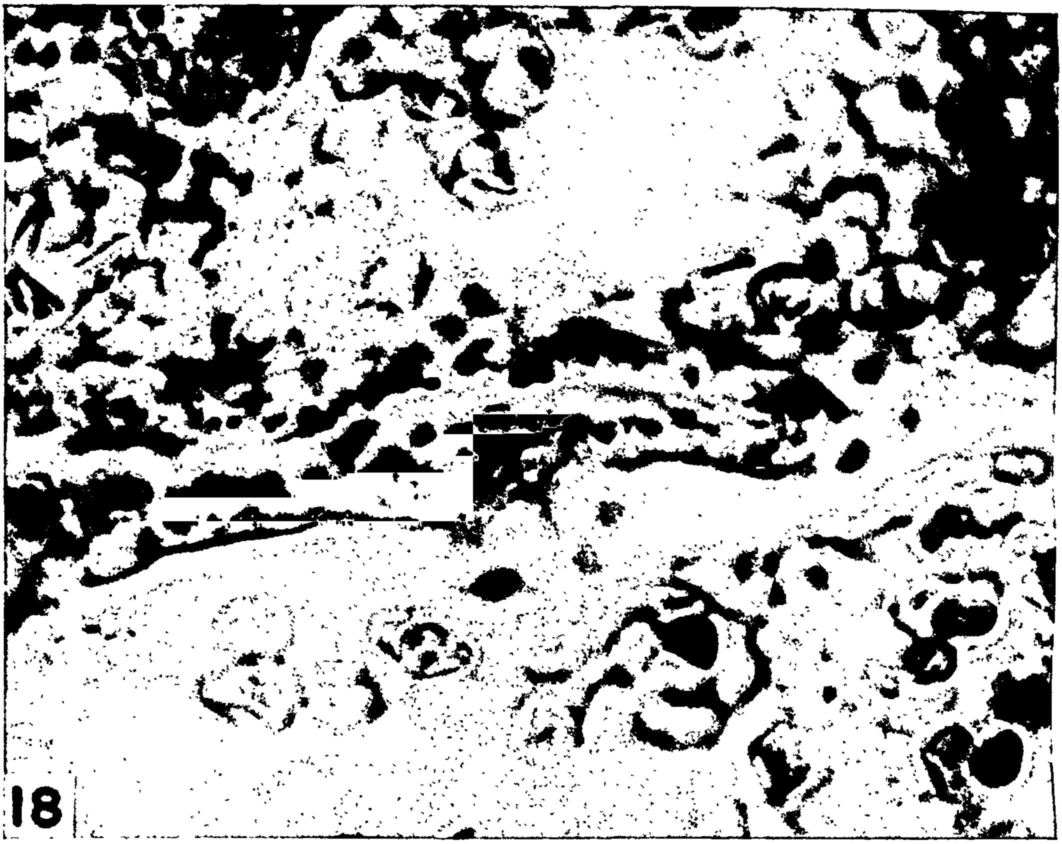

PLATE VII

FIG. 18. Rat placenta (near term). A chorionic villus surrounded by maternal blood. The syncytial cells contain blue fat droplets located on the fetal side of the cells. Photographed through a red filter. ×2000.

and are deomonstrated by the Schiff reagent (Feulgen and Voit, 1924). Plasmalogens have been demonstrated by this series of reactions in these glands and other tissues (Feulgen and Bersin, 1939). The structure of the plasmalogens was established by isolation of the aldehydes from muscle (Anchel and Waelsch, 1942). They are phospholipids in which the aldehydes are bound to glycerol through an acetal linkage and which, in addition, carry a phosphate ester of ethanolamine (Tannhauser and Schmidt, (1946). Since aldehydes react readily with hydrazines, the specificity of the methods for ketosteroid depends upon whether hydrolysis of plasmalogens occurs under the conditions given. The demonstration of plasmalogens in tissue by the Schiff reagent requires prior treatment with mineral acid or mercuric chloride, unless the Schiff reagent is itself sufficiently acid to produce hydrolysis. It is claimed that exposure of tissue to formalin for more than six hours will also hydrolyze the acetal and that further exposure to formalin will destroy the plasmal that has been liberated (Hayes, 1949). In the case of ketosteroid, formalinization of tissue is

necessary to unmask the carbonyl group. Once this is accomplished (one or more hours) deterioration of the staining reaction with further exposure to formalin (weeks) does not occur. This is in contrast to what is claimed for plasmal (Hayes, 1949).

Although the phenylhydrazine reaction occurs with both aldehydes and ketones, the Schiff reagent reacts readily with aldehydes, less readily with certain cyclic ketones (Karrer, 1938), presumably with easily oxidizable $\alpha$-ketols, and not at all with most ketones. The Schiff reagent has been reported to react *in vitro* with pure steroids (Dempsey and Wislocki, 1944; Dempsey, 1946). However, others claim that ketosteroids failed to react with the Schiff reagent (Albert and Leblond, 1946; and Oster and Oster, 1946). The four monoketosteroids used above for studying the hydrazine reaction failed to react with the Schiff reagent. This would suggest that the Schiff reagent demonstrates aldehyde or possibly $\alpha$-ketol, and that the hydrazine reaction can demonstrate aldehyde and ketosteroid. It is claimed that the Schiff reaction occurs in exactly the same locations as the hydrazine reaction (Albert and Leblond, 1946). In pilot experiments with adrenal gland of rabbit and ovary of dog, in which the period of fixation in neutral formalin was varied from 15 minutes to weeks, it was found that the blue stain developed with the hydrazide reaction was maximal after 1 hour's fixation and did not change significantly in intensity one way or another after longer fixation; nor did the location of the reaction change. No reaction with the Schiff reagent occurred with adjacent sections fixed for a short time or for very long periods. Other sections treated with mercuric chloride for 5–10 minutes gave the same degree of staining or slightly more intense staining with the hydrazide, and in some sections of unfixed adrenal gland so treated gave a blue color only in the reticularis and medulla where ketosteroid is rarely found. Mercuric chloride did not unmask ketosteroid in the way that formalin fixation did. Sections fixed in formalin for 4 hours and then stained were less blue than similar sections treated with mercuric chloride. In the latter, reticularis and medulla were also blue. Furthermore, plasmal has been isolated from skeletal muscle (Anchel and Waelsch, 1942) but the carbonyl reaction with the hydrazide gave consistently negative results with formalin-fixed muscle.

Proof of the specificity of the hydrazine reaction for ketosteroid, when conducted as herein described, hinges upon whether the carbonyl groups are aldehydic or ketonic. Reactions which depend upon the relative reactivity of aldehydes and ketones with reagents such as Schiff, cannot settle the question definitely. Conclusive proof would be afforded by a reaction which depended upon whether the carbonyl group carried a hydrogen atom ($\mathrm{H}\underset{\underset{\displaystyle \mathrm{O}}{\|}}{\mathrm{C}}\text{—R}$) or only carbon ($\text{R—}\underset{\underset{\displaystyle \mathrm{O}}{\|}}{\mathrm{C}}\text{—R}$),

rather than upon the reactivity of the carbonyl group itself. Such a reaction is available. The phenylhydrazones of aldehydes couple with diazonium compounds in pyridine (Seligman, Gofstein, and Rutenburg, 1949) to form colored water-insoluble formazans. The phenylhydrazones of ketones cannot undergo the reaction because of the absence of replaceable hydrogen. Utilizing this reaction and tetrazotized diorthoanisidine, a blue (benzaldehyde, furfural) or dark red (formaldehyde, butyraldehyde) diformazan is formed from the appropriate phenylhydrazone. This new histochemical reaction for aldehydic carbonyl groups forms the subject of another communication (Seligman and Ashbel). Preliminary experiments with this method suggest that free aldehydes are not present in adrenal cortex fixed in neutral formalin for 24 hours and not exposed to strong hydrolytic agents.

It may, therefore, be concluded that the hydrazide reaction with carbonyl groups of lipoid demonstrates ketosteroid in tissue, after prior fixation in formalin from 1 hour to many weeks.

## SUMMARY

A new method for demonstrating carbonyl groups in the lipoid of formalin-fixed tissues is described. The method is based on the reaction of aldehydic and ketonic groups with 2-hydroxy 3-naphthoic acid hydrazide followed by coupling of tetrazotized diorthoanisidine into the naphtholic ring with the production of a blue, insoluble azo compound. Reaction with certain carbonyl groups of non-lipoid material in nervous tissue, elastic tissue, and reticulum is described. Carbonyl-reacting lipoid was found in adrenal cortex, corpus luteum, interstitial cells of testis and syncytium of placenta. The localization in tissue was similar to that previously demonstrated with other methods. Evidence is presented that the carbonyl-containing lipoid in these formalin-fixed glands is in fact ketosteroid.

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# A NEW REAGENT FOR THE HISTOCHEMICAL DEMONSTRATION OF ACTIVE CARBONYL GROUPS. THE PREPARATION OF 2-HYDROXYNAPHTHALENE CARBOXYLIC AND SULFONIC ACID HYDRAZIDES[1]

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In order to develop a histochemical method for active carbonyl groups, which would result in the production of intensely colored, insoluble dyes, acid hydrazides of several naphthols were prepared. It was expected that coupling of a suitable diazonium compound into the naphthol, after reaction of the hydrazide group with carbonyl, would yield azo dyes of suitable intensity for microscopic delineation.

THE hydrazides of three hydroxy naphthoic acids and 2-hydroxy-6 naphthalene sulfonic acid were prepared. The sulfone hydrazide failed to react with ketosteroid in adrenal cortex under conditions in which the carboxylic acid hydrazides did react (Ashbel and Seligman, 1949). The pigment produced on coupling with diazotized diorthoanisidine, after reaction of the three naphthoic acid hydrazides with ketonic material, was blue (2-hydroxy-3-naphthoic acid hydrazide), brownish purple (1-hydroxy-2-naphthoic acid hydrazide), and violet (2-hydroxy-6-naphthoic acid hydrazide). The first pigment was most suitable for histochemical study and the hydrazide was readily prepared. The preparation of the four hydroxy hydrazides is described below. In addition the preparation of 2-naphthoic acid hydrazide is given. This material was used for control studies (Ashbel and Seligman, 1949).

## EXPERIMENTAL[2]

### *2-Hydroxy-3-naphthoic acid hydrazide*

The acid chloride was prepared from 20 gms. of 2-hydroxy-3-naphthoic acid (Eastman technical grade) by warming with 25 cc. of thionyl chloride until all the solid had dissolved and hydrogen chloride

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[1] This investigation was aided by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Microanalyses by Shirley Katz. All melting points are corrected.

was no longer evolved (Meyer, 1901). The excess thionyl chloride was removed by distillation at reduced pressure. The acid chloride solidified on cooling and dissolved with the evolution of heat upon addition of 50 cc. of absolute methyl alcohol. The alcohol was removed by distillation at reduced pressure. To the low melting ester that was formed, was added 35 cc. of 85 per cent hydrazine hydrate and the mixture was heated on the steam bath for 3 hours (Franzen and Eichler, 1908). The reaction period was shortened by use of 100 per cent hydrazine hydrate. The hydrazide separated from the reaction mixture as a crystalline mass on cooling. Water was added, the crystals were collected with the aid of suction and washed with water. The crystals were dissolved in 500 cc. hot alcohol. After treatment with active charcoal, the solution was chilled and the crystals collected and washed with ether. The pale-yellow platelets melted at 203–4°. The yield was 16 gms. (75 per cent). The reported melting point is 203–4° (Franzen and Eichler, 1908).

*1-Hydroxy-2-naphthoic acid hydrazide*

This hydrazide was prepared from 1-hydroxy-2-naphthoic acid (Eastman), exactly as described for the 2, 3 isomer above. It was obtained as fine, pale-tan needles, melting at 212–213°. The same melting point is given by Beyer and Schulte, 1941.

*2-Hydroxy-6-naphthoic acid*

Attempts to prepare 2-hydroxy-6-naphthoic acid according to the method of Howarth and Sheldrick (1934) gave poor results. This material was prepared from the nitrile by hydrolysis with hydrobromic acid in acetic acid. 2-Hydroxy-6-bromonaphthalene was prepared from beta naphthol (Organic Syntheses, 1940) and methylated with methanol and sulfuric acid (Woodward and Eastman, 1944). A mixture of 90 gms. of 2-methoxy-6-bromonaphthalene and 60 gms. of copper cyanide was heated for one hour at 240°C. The mass dissolved and became brown. The liquid was poured onto a cold surface and pulverized. The powder was warmed in a solution of 300 cc. of concentrated ammonia in 1 liter of water. The mixture was filtered and the residue was distilled at reduced pressure. The nitrile was obtained in 65–70 percent yield, b.p. 205–8°, 14 mm.; m.p. 103°. This material (14 gm.) was heated under reflux overnight with 30 cc. of glacial acetic acid and 100 cc. of 48 per cent hydrobromic acid. Upon cooling, the hydroxy acid crystallized. It was dissolved in ethanol, treated with norite, and after the addition of water, crystallized as tan needles melting at 241°; yield 11 gms. (78 per cent).

*2-Hyrdoxy-6-naphthoic acid hydrazide*

The ethyl ester was prepared by solution of 1.5 gms. of 2-hydroxy-6-naphthoic acid in 15 cc. of absolute ethanol and by saturation of

the solution with dry hydrogen chloride. After standing for 12 hours the mixture was diluted with water. The oil that separated crystallized slowly on standing. The crude ester was warmed on the steam bath with 2 cc. of 85 per cent hydrazine hydrate for 1 hour, at which time the hydrazide had separated as a white crystalline solid. After recrystallization from alcohol, the product melted at 256–8°; 0.5 gm. A second crop 0.3 gm. (m.p. 254–6°) was obtained by concentration of the mother liquor.

Analysis calculated for $C_{11}H_{10}N_2O_2$: C, 65.35; H, 4.96
Found: C, 65.33; H, 4.96

*2-Hydroxy-6-naphthalene sulfonhydrazide*

The required 2-carbethoxy-6-naphthalene sulfonic acid was prepared (Zincke and Dereser, 1918) by the slow addition of 21 cc. of ethyl chlorocarbonate to a vigorously stirred solution of 49.2 gms. of sodium 2-hydroxy-6-naphthalene sulfonate (Eastman technical grade) and 8 gms. sodium hydroxide in 200 cc. of water. The stirring was continued for an additional hour, after which the mixture was cooled to precipitate the carbethoxy derivative. This material was collected with the aid of suction and dried by warming on the steam bath. The crude product (44 gms.) was ground with an equal weight of phosphorus pentachloride and was heated on the steam bath for 2 hours (Zincke and Dereser, 1918). The excess phosphorus pentachloride was decomposed by grinding the fused melt with ice. The acid chloride was filtered and after recrystallization from acetic acid melted at 116–18°, 15 gms.

The carbethoxy acid chloride (1 gm.) was heated for an hour on the steam bath with 5 cc. of 85 per cent hydrazine hydrate in which it dissolved. The solution was evaporated to dryness under reduced pressure and the residue was extracted with boiling methanol. After concentration and cooling, fine white needles were obtained. After two crystallizations from methanol the melting point without decomposition was 188°; yield 0.5 gm. (65 per cent).

Analysis calculated for $C_{10}H_{10}O_3N_2S$: C, 50.41; H, 4.19
Found: C, 50.46; H, 4.09

*2-Naphthoic acid hydrazide*

Beta naphthoic acid (10 gms.) was converted to the acid chloride, the methyl ester, and finally the hydrazide exactly as described for 2-hydroxy-3-naphthoic acid hydrazide. The product crystallized from alcohol-water in white needles melting at 146–8°; yield 6.8 gms. (62 per cent). The reported melting point is 147.5° (Goldstein and Cornamusaz, 1932).

## SUMMARY

The preparation of five acid hydrazides is given. 2-Hydroxy-3-naphthoic acid hydrazide was found to be the most convenient to

prepare, and to be the most suitable for the histochemical reaction with steroidal carbonyl groups.

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# NOTES AND COMMENTS

## 1-METHYL-2-MERCAPTOIMIDAZOLE: AN ANTITHYROID COMPOUND HIGHLY ACTIVE IN MAN[1]

THE antithyroid compounds used for the treatment of hyperthyroidism were previously selected for clinical trial on the basis of high potency in the rat (Astwood, Bissell and Hughes, 1945). Subsequent experience, showing that the rat assay was a poor index of effectiveness in man, led to the de-

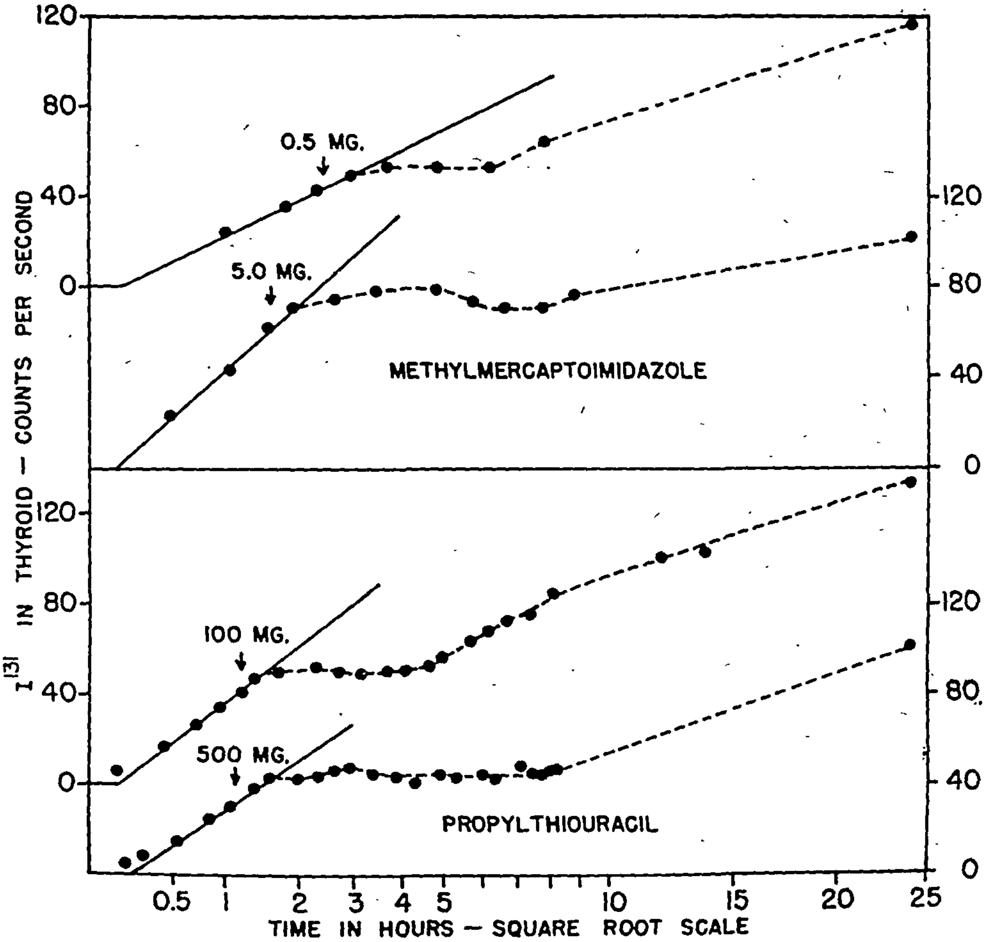


FIG. 1. Comparison of the effects of two doses, 0.5 and 5.0 mg. of 1-methyl-2-mercaptoimidazole with two doses, 100 and 500 mg., of propylthiouracil on the course of the iodine uptake by the thyroid gland in normal human subjects. Five-tenths mg. of 1-methyl-2-mercaptoimidazole was somewhat less effective than 100 mg. of propylthiouracil, while 5 mg. of the former exerted a more sustained effect than 500 mg. of the latter. The ordinate is an arbitrary scale of counts per second and the abscissa is the square root of the elapsed time after the oral administration of 100 microcuries of $I^{131}$. On this plot, a deviation from the normal straight line course of accumulation during the first 8 or 10 hours indicates inhibition.

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TABLE 1. EFFECT OF VARIOUS DOSES OF ANTITHYROID COMPOUNDS ON THE $I^{131}$ UPTAKE BY THE THYROIDS OF NORMAL HUMAN BEINGS

| Compound | Degrees of response* | | | | | | | | | Relative activities (thiouracil = 1.00) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dose in milligrams | | | | | | | | | | |
| | 0.5 | 1 | 2.5 | 5 | 10 | 25 | 50 | 100 | 500 | Man | Rat† |
| 2-Thiouracil | | | | | | 1 | 2, 3 | 3, 4, 4 | | 1.0‡ | 1.0 |
| 6-Methylthiouracil | | | | | | 2 | 3, 4 | 4 | | 2.0‡ | 1.0 |
| 6-n-Propylthiouracil | | | | | | | 1, 1, 2 | 3, 3, 3 3, 3 | 4 | 0.75‡ | 11.0 |
| 2-Mercaptoimidazole | | | | 0, 1, 2 2, 2, 3, 3 | 4, 4 2, 3, 4 | 4, 5 | | 5 | | 10.0‡ | 2.0 |
| 1-Methyl-2-mercapto-imidazole | 3, 3, 3 | 2, 4 | 4 | 4, 5 | 4, 5 | 5, 5 | | | | 100.0 | 2.0 |

* The degrees of response were classified as follows: 0 = no effect, 1 = slight or questionable effect, 2 = distinct but incomplete inhibition, 3 = complete inhibition for less than 4 hours, 4 = complete inhibition for more han 4 but less than 24 hours, 5 = complete inhibition for 24 hours or longer.
† Reference 2 and unpublished data.
‡ Reference 1.

velopment of a method using $I^{131}$ by which these substances were tested directly in normal human beings (Stanley and Astwood, 1947).

Antithyroid activity determined in this way correlated quite well with clinical effectiveness. 2-Mercaptoimidazole, which was found to be about ten times as active as thiouracil, has now been used to treat thirty-four patients during a period of two years. The results obtained were quite in keeping with the predictions of its potency based on the iodine uptake test.

During the course of testing a limited number of further compounds by the radioiodine method, it was observed that 1-methyl-2-mercaptoimidazole was remarkably active. As little as 0.5 Mg. exerted a pronounced inhibitory effect on iodine accumulation and doses of 5.0 Mg. completely inhibited the uptake for nearly twenty-four hours (Fig. 1). In Table 1 are shown the various grades of inhibition induced by different doses of this compound in comparison with various doses of thiouracil, methylthiouracil, propylthiouracil, and 2-mercaptoimidazole. From these data it was estimated that the activity of 1-methyl-2-mercaptoimidazole was approximately 100 times that of thiouracil.

Preliminary studies on thirty patients with hyperthyroidism treated with this compound have confirmed a high degree of effectiveness.

ACKNOWLEDGMENTS

The 2-mercaptoimidazole was generously supplied by Dr. R. O. Roblin and associates of the Stamford Research Laboratories of the American Cyanamid Company. We are indebted to Dr. D. C. Hines of the Eli Lilly Research Laboratories for the 1-methyl-2-mercaptoimidazole used in these studies.

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# SAMPLE ABSTRACTS

BEATH, O. A., C. S. GILBERT, and H. F. EPPSON. (Wyoming Agric. Expt. Sta., Laramie.) / The use of indicator plants in locating seleniferous areas in western United States. II. Correlation studies by states. / Amer. Jour. Bot. / 26(5); 296-315. 1939.—Studies were made of the Se content of 563 specimens of native plants from 12 of the western states, correlating the presence of Se in plants with the geological formation in which the plants grew. Special attention was given to spp. of Stanleya, Oonopsis, Xylorrhiza and Astragalus as primary indicators of Se in formations contemporaneous with known seleniferous formations of Wyoming and also in formations not previously investigated. The Stanleya and Astragalus spp., because of their wide distribution, proved especially valuable as indicators. The following are added to the list of known seleniferous formations: Pennsylvania limestones of southeast and central Nevada; Payette Lake sediments in southwest Idaho; Carbonaceous and limy shales in Provo Canyon, Utah; Tertiary sediments in Tonto Basin, Arizona; White Tank Monzonite near Twenty-nine Palms, California; the Monkopi formation in southern Utah and northern Arizona; and certain areas in southeast Idaho, northwest Utah, southeast Oregon and Nevada.—O. A. Beath.

DeSMIDT, F. P. G. (Med. Res. Lab., Kenya.) / Rapid production of autogenous vaccine for treatment of pneumonia. / Brit. Med. Jour. / 1938(4065): 1140-1142. 1938.—An autogenous vaccine made directly from mouse peritoneal washings ("M. P. W." vaccine) can be ready in less than 12 hours, as compared with several days required by the usual method for preliminary typing, growth, isolation of colonies and subsequent sterility tests. DeSmidt trusts to the dominance of the type of pneumococcus inj. into the mouse, to the disappearance in the mouse's body of other bacteria which may have been in the sputum, and to strictly aseptic technic throughout to secure pure, sterile vaccine. His procedure is as follows: Inoculate 2 mice intraperitoneally with 0.5 ml. of rather thick saline sputum emulsion; chloroform the mice when moribund—do not allow them to die; take utmost precautions for sterility of board, tools, skin and abdominal wall before opening peritoneal cavity; Gram-stain a smear of peritoneal exudate for verification; make a preliminary washing of peritoneal cavity into 1 ml. of plain saline for bile solubility and agglutination tests (this preliminary wash may be omitted), make peritoneal washing in 4 ml. of 0.5% phenol saline into sterile centrifuge tube with sterile stopper; centrifuge at 6000 r. p. m. for 30 sec., pipette supernatant pneumococcus susp. into sterile cotton-plugged tube; cover with rubber cap; immerse in water-bath at 60° for 30 min.; place the killed susp. in vaccine bottle. The Burroughs Wellcome opacity tube is used to estimate the strength of suspensions, usually 1,000 to 3,000 millions per ml. Dose 1,000 millions of cocci in 0.5 ml. The vaccine may be administered immediately after bottling, the usual sterility tests being dispensed with—J. B. Paton.

STEFANELLI, A. (U. Rome.) / Il metabolismo dell'uovo e dell'embrione studiato negli anfibi anuri. II. L'assunzione di ossigeno. [Metabolism studies on amphibian (anuran) eggs and embryos. II. The oxygen intake.] / Arch. Sci. Biol. [Naples] / 24: 411-441. 1938.—By a microrespirometric method the $O_2$ uptake of individual eggs of Bufo vulgaris and B. viridis was detd. before and after fertilization (which was carried out in the manometer), gastrulation, and neurulation. The $O_2$ consumption increased during gastrulation and after completion of the neurulation; after that it ran proportional to the number of cells until absorption of the yolk was complete. Equal wts. of eggs, regardless of individual egg size, take up equal amts. of $O_2$ during equal periods of time or development. The faster developing B. viridis consumes more $O_2$ per unit of time, but the same amt. as B. vulgaris for the same degree of development.—H. Sobotka.

ECKSTEIN, K. (Forstliche Hochschule, Eberswalde.) / Die "Abbrüche" des Waldgärtners, Myelophilus piniperda L. / Fortwiss. Centralbl. / 61(2): 33-42; (3): 81-92. 4 fig. 1939.—The larva bores into pine twigs and hollows them out so that they eventually break off. 50 to 150 twigs may be lost by a single tree during a season. Similar damage is done by M. minor and Pityophthorus glabratus.—W. N. Sparhawk.

POĬUROVSKIĬ, S. D. (Inst. Usovershenstvovaniia Vracheĭ, Leningrad.) / [Photoelectrometric determination of protein fractions of blood serum.] / [In Russ. with Ger. summ.] Biokhimiia / 3(6): 713-722. 1938.—Photoelectric detn. of blood serum proteins can be conducted on samples as small as 0.1 ml. with an error of 2% or less. In a sample diluted 1:100, the globulin fraction is detd. first by half-saturation with $(NH_4)_2SO_4$ and comparing the light absorption of the suspension with that of a prepared blank. The sample is filtered and the albumin fraction in the filtrate detd. by addition of 5% sulfosalicylic acid and again comparing with a blank. The readings are converted to percentages by means of tables.—E. K. Johnson.

MENKIN, VALY. (Harvard U.) / Études sur l'inflammation. / Rev. Immunol. [Paris] / 5(2): 113-137. 1939.—In discussing the mechanism of the inflammatory reaction and its rôle in immunity, the author reviews his many previous studies. The inflammatory process tends to localize and ultimately to destroy the irritating factor. This leads to repair. The exudation of plasma, detd. by a rise in intracapillary pressure and an increase in capillary permeability, is due to a crystalline, non-protein, diffusible substance liberated by the injured tissues. The so-called "leukotaxine" is not histamine, but seems to be a polypeptide. It is thought to be responsible also for the migration of leucocytes to the inflamed region. As the inflammatory process progresses, lymphatic drainage is blocked by thrombi in the lymphatic channels and coagulated plasma in the injured site. The fixing capacity of an inflammation is proportional to the injury exerted by the irritant. The irritants that cause necrosis produce a rapid local reaction which neutralizes their power of dissemination. The contrary is true of weak irritants. This explains the action of the pyogenic bacteria, their rapid invasion and the rôle of the inflammatory reaction in the development of immunity and host resistance. The walling off of the inflamed area tends to produce a local acidity that determines the cytology of the region. Polymorphonuclear leucocytes persist in an exudate whose pH is above 7; if the pH falls to 6.8 or 6.9, mononuclears predominate; in an exudate with pH of 6.5 or less, all types of cells are destroyed.—W. I. Kelly.

CURTIS, JOHN T. (U. Wisconsin.) / The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. / Amer. Jour. of Bot. / 26(6): *390-399. 9 fig. 1939.*—Ten spp. of Rhizoctonia were isolated from 23 spp. of orchids from various habitats in the U. S. and Central America. Two new species and a new var. are descr.: R. borealis, from Goodyera repens var. ophioides and Spiranthes gracilis, Wisconsin; R. subtilis var. nigra, from S. cernua, Wisconsin; and R. moniliodes, from Habenaria dilatata, H. hyperborea, Liparis liliifolia, Pogonia ophioglossoides and S. romanzoffiana, Wisconsin, and Sobralia pleiantha, Costa Rica. No evidence of specificity was found, as one orchid species could harbor several fungus spp. and any one fungus sp. could attack several orchid spp. The fungi were correlated with ecological habitat rather than with orchid spp. Symbiotic germination tests, using fungi isolated from a given orchid in combination with the seeds of that orchid, indicated in most cases that the fungi were unable to induce seed germination. The symbiotic relationship is considered to be one of parasite and host, with the orchid deriving no benefit from the fungus in its roots.—J. T. Curtis.

## Abbreviations

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| alpha | α | distribution | distr. | lethal dose, minimum lethal dose | M.L.D. | percent | % |
| amount | amt. | experiment(al) | expt., exptl. | male, males | ♂, ♂♂ | pound(s) | lb., lbs. |
| approximately | approx. | female, females | ♀, ♀♀ | microgram(s) | γ | second(s) | sec. |
| beta | β | foot, feet | ft. | milligram(s) | mg. | solution | soln. |
| concentration | conc. | gamma | γ | milliliter | ml. | species | sp., spp. |
| cubic centimeter | ml. | gram(s) | g. | minute(s) | min. | temperature | temp. |
| described | descr. | hour(s) | hr., hrs. | ounce(s) | oz. | ultra-violet | u.-v. |
| determined | detd. | inch(es) | in. | | | variety(ies) | var., vars. |
| | | | | | | volume(s) | vol. |

*Use chemical symbols for the elements and the simpler inorganic compounds.*

# NEW BOOKS

Annual Review of Physiology, Volume XI, 1949, ix+643 pp. Annual Reviews, Inc. Stanford, California. $6.00.

The present volume begins the second decade of life for the Annual Reviews of Physiology. As compared with human chronology, it might therefore be regarded as pre-adolescent, a period of great stress and strain, and characterized by rapid growth and awkward locomotion. The analogy is perhaps not too far-fetched, since the Editors in their preface note that the current reports in physiology are increasing in geometric progression and that their task in preparing the annual volume is one requiring continuous adaptation. One can sympathize with them in their labors, the more so since the volume defies reviewing by any of the ordinary artifices. For how can one review a volume written by twenty-two different authors, covering the entire field of physiology, condensed to a degree that each sentence summarizes itself an entire article or series of articles, and even then compressed still further so that one author plaintively remarks that he read 1000 articles only to end by reviewing 288 of them?

The task, then, of preparing such a volume is formidable, and it is impossible to compare its form, substance, literary quality and scientific validity with those properties of similar books. One might question whether the effort is worth the trouble. Indeed, considering the amount of condensation represented in such a volume, one can ask whether it performs any significant function not provided by the Index and Abstract periodicals. There seems to be danger that the Annual Reviews may become merely a list of the articles published during the year, for the reviewers, more and more in recent times, are falling into what might be called the Annual Reviews style, in which each sentence begins: "Doe and Everyman (1948) found that . . . ." The Editors are obviously aware of this tendency and remark that they have "earnestly pleaded with our reviewers to consider only those papers which they deem particularly noteworthy contributions to our science and to reduce coverage of other papers to mere listing."

It seems ridiculous for productive investigators to spend the time necessary for mere listing of unimportant papers. Such lists are available, and better done, in the Index journals. It also seems unwise to restrict coverage to the papers published in a single year, for by so doing concepts are taken out of historical context. It moreover seems unprofitable for a reviewer, striving for completeness, to use space and energy on topics not directly related to his own interests and experience. On the contrary, a better policy would be to write with the greatest possible insight on a few topics, indicating their historical development, a critical analysis of their present status and a projection into the future of their probable development. Such reviews are the proper concern of investigators. They represent the thought out of which progress is made. No apology need be made for lack of completeness—in a decade each field would be considered by ten of its leading exponents. If the thoughts of its leaders do not define the subject material of a field, what does?

Natural Products Related to Phenanthrene. 3rd Edition, 1949. Louis F. Fieser and Mary Fieser. American Chemical Society Monograph No. 70. Reinhold Publishing Corporation, 330 W. 42nd St., New York, N. Y. xii+704 pp. $10.00.

The continuing interest in the chemistry of the steroids has led to a vast amount of work since the 2nd edition of this book was published in 1937. The present edition is, therefore, more nearly a new book than an ordinary revision. Appropriate recognition of stereochemical information has been accomplished by the addition of a special chapter by Dr. Richard B. Turner. The large amount of literature relating to partial and total syntheses has been carefully summarized. Considerable attention has been devoted to new methods for characterizing steroid compounds, including optical rotation data, infrared and ultraviolet absorption spectra and x-ray diffraction. Last but not least, the systematic consideration of the field has compelled the authors to come to grips with the nomenclature problem. The result is a new systematic nomenclature which departs from the older schemes only where consistency demands it.

Quite aside from the great virtues of this book as a summary of purely chemical information, it is of great importance to physiological endocrinology. Indeed, it is monolithic in character, for were no other reference available, it would provide an astonishingly complete account of the known information about the steroid-secreting endocrine glands. Not the least of its accomplishments is the avoidance of questionable data and controversial interpretations regarding the regulation of endocrine organs. Fact and fancy are so promiscuously intermixed in the literature of reproductive and adrenal physiology that it takes rare ability to cleave through the claims and counter claims and arrive at a balanced estimate of the total situation.

A word, at least, should be said to compliment the lucid, unobtrusive and generally excellent literary style of the authors. The book is nicely printed with a legible typeface on good quality paper. Profuse use has been made of tabular material and of structural formulas. Documentation is extensive, the references being set as footnotes for the convenience of the reader. The volume is completed by adequate and accurate author and subject indexes. It is a pleasure to commend this admirable monograph to the attention of everyone interested in endocrinology.

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# SUBJECT INDEX, VOLUME 44